REMARKS

Claims 1, 3, 14, and 43-50 constitute the pending claims in the present application.

Applicants' agent and the Examiner conducted an Interview on March 17, 2005. Applicants with to express gratitude to the Examiner for her constructive arguments, comments and suggestions, as well as her patience. The following remarked are made in view of the Interview.

Applicants respectfully request reconsideration in view of the following remarks.

Claim rejections under 35 USC §112, Second Paragraph

Claims 1, 3, 14, and 43-50 are rejected under 35 USC 112, second paragraph, for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter Applicants regard as the invention. Specifically, the Office Action rejects the term "characterized," since it allegedly is unclear whether the limitation(s) following the phrase are part of the claimed invention.

Applicants have amended Claim 1 to clarify the subject matter claimed, thereby obviate this rejection. The amendment does not narrow the scope of the claim. Reconsideration and withdrawal of the rejection are respectfully requested.

Claim rejections under 35 USC §103(a)

Claims 1, 3, 14, 43-49 are rejected under 35 U.S.C. 103 as being unpatentable over Girasole *et al.* in view of Kishimoto *et al.* (U.S. Pat. No. 5,888,510), for the reasons of record set forth at pages 2-4 of the previous Office Actions (3/18/04 and 5/25/04).

Prior to the Interview on March 17, 2005, for the purpose of having a more productive interview, Applicants submitted to the Examiner a draft response with a Rule 132 Declaration (unsigned) by inventor Shaughnessy. In view of the draft response and the unsigned Declaration, the Examiner requested Applicants to provide explicit support for the amended Claim 1.

Therefore, Applicants will first address the support issue raised by the Examiner during the Interview, then address the rejection in the pending Office Action.

First of all, Example 2 demonstrates that treatment of OVX mice (*in vivo* experiment) with

the claimed IL-11 Ab results in **both** increased bone formation **and** decreased bone resorption. Specifically, page 25, second full paragraph indicates that IL-11 Ab treatment results in "**higher rates of bone formation**," as measured by osteoid surface measurements (See Figure 6B. Also see page 1, lines 12-13: "[o]steoblasts are responsible for the formation of new bone osteoid"). Meanwhile, page 25, third full paragraph indicates that IL-11 Ab treatment results in "**a notable reduction**" of osteoclast activity, as measured by osteoclast surface measurements; and "**much less bone resorption**" in IL-11 Ab treated animals. See Figure 6C. In addition, page 25, first full paragraph indicates that IL-11 Ab treatment leads to a result in which "**cancellous bone loss was not only prevented but that it was reversed by the inhibition of biological activity of IL-11.**"

These *in vivo* data unequivocally show that IL-11 antagonist treatment of OVX mice (animal model of osteoporosis) not only inhibits osteoclast / bone resorption, but also stimulates osteoblast / bone formation in the same animal, resulting in prevention of bone loss, or even a net gain in bone mass. Thus the claimed subject matter is fully supported by the specification.

Applicants also wish to bring the Examiner's attention to the following disclosure regarding the claimed disease conditions.

On page 4, second full paragraph, the specification indicates that: "[a]ccordingly, the present invention from one broad aspect provides a process for treating or alleviating the symptoms of a pathological condition in which bone density is decreased...Such conditions include those involving increased bone resorption **and** lack of desirable bone formation." (emphasis added)

Also on page 3, lines 24-27: "[i]t is an object of the present invention to provide a novel procedure for treatment and for alleviation of the symptoms of clinical conditions, such as osteoporosis, in which increased bone resorption **or** decreased bone formation is the underlying pathology." (emphasis added)

The follow section of this response addresses the issues raised in the Office Action.

In the previous response, Applicants have advanced the following arguments:

1) unlike Rheumatoid Arthritis, bone density is controlled *in vivo* by two types of counter-acting cells, osteoclast and osteoblast. The *in vitro* results of Girasole only teaches the effect of IL-11 signaling on osteoclast, but is completely silent about its effect on osteoblast. More importantly, it is completely silent about the unexpected discovery that IL-11 signaling has opposite effects in osteoclast and osteoblast *in vivo*, e.g., inhibiting IL-11 function simultaneously inhibits osteoclast function *and* enhances osteoblast function;

2) IL-11 signaling may not be significant enough to have an *in vivo* effect on bone density;

3) There is no motivation for a skilled artisan to predict IL-11 *in vivo* results from the results of an unrelated protein (IL-6) as described in Kishimoto.

Especially for point 1) above, Applicants submit that there is simply nothing in the cited references that would teach or suggest to a skilled artisan that IL-11 would have opposite effects in two different type of cells (osteoclast and osteoblast) – in contrary, conventional wisdom would strongly suggest that the same cytokine (IL-11) would have the same or similar effects on different cell types (promoting osteoclast function – which is reducing bone density; and promoting osteoblast function – which is increasing bone density).

The instant Office Action asserts that "this property of IL-11 signaling" (*e.g.* having **opposite** effects on osteoblast and osteoclast function) is "inherent in the prior art process, *i.e. in vivo* there is a balancing process, and inhibiting IL-11 function would (necessarily) simultaneously inhibit osteoclast function and enhance osteoblast function." In other words, the Office Action reaches this conclusion by assuming without proof, that there is a "see-saw" type of balancing effect *in vivo* in terms of osteoclast and osteoblast function, such that anything that affects the bone resorption function of osteoclast in one direction would necessarily affect the bone formation function of osteoblast in the opposite direction. This is scientifically unsound, and Applicants respectfully disagree.

Applicants hereby submit a Rule 132 Declaration by inventor Dr. Stephen Shaughnessy, which Declaration directly contradicts the Office Action position above. The cited references are also enclosed herewith as **Exhibits A-J**.

First of all, in direct contradiction to the Office Action position, paragraph 10 of the Declaration indicates that in ovariectomization, a pathological condition similar to post-menopausal osteoporosis, **both** bone resorption and bone formation are increased. This would not be true if the see-saw type of "equilibrium" as asserted in the Office Action is present. Paragraph 10 is supported by three references published before the filing of the instant application.

Secondly, in direct contradiction to the Office Action position, paragraph 11 of the Declaration indicates that many agents (*e.g.* estrogen, bisphosphonates, and calcitonin) simultaneously affect **both** osteoblast and osteoclast function in the same direction (*e.g.* inhibition). This too would not be true if the see-saw type of "equilibrium" as asserted in the Office Action is present. Paragraph 11 is supported by five references published before the filing of the instant application. Reference v (Kimble) indicates that an agent (*e.g.* IL-1R antagonist) may inhibit (osteoclast-mediated) bone resorption, while having no effect on (osteoblast-mediated) bone formation. This further demonstrates that osteoclast and osteoblast functions are independently regulated. Thus it is completely novel and unobvious that one agent (*e.g.* IL-11 Ab) can have completely opposite effects on osteoclast and osteoblast functions.

Paragraph 12 of the Declaration further contradicts the Office Action position by providing evidence that some agents (*e.g.* PTH) simultaneously affect **both** osteoblast and osteoclast function in the same direction – in this case, stimulation. Again, this would not be true if the see-saw type of balancing is at work, and this further demonstrates that osteoblast and osteoclast functions are independently regulated.

Therefore, a skilled artisan, in view of Girasole and this body of work regarding regulation of osteoblast and osteoclast function, would have no clue as to the effect of IL-11 antagonist on the function of osteoblast, either *in vitro* or *in vivo*. Girasole only studies the effect of IL-11 signaling in osteoclast, but is completely silent about the effect of IL-11 signaling in a very different, and independently regulated osteoblast. In other words, there is simply no teaching or suggestion in the prior art or common knowledge regarding the effect of IL-11 antagonists (*e.g.* IL-11 Ab) on osteoblast function, either *in vitro* or *in vivo*. Without this, there is simply nothing to be combined with Girasole to reach the claimed invention. It naturally follows that a skilled artisan would have had no reasonable expectation of success to arrive at the claimed invention.

Even if in view of the fact that there are IL-11 receptors on both cell types, a skilled artisan would at best make a logical and educated guess that IL-11 signaling would have similar (rather than opposite) effects in both cell types, which surprisingly turns out to be wrong. This further demonstrates the surprising finding of the inventors, upon which the claimed invention is partially based.

Neither does Girasole inherently teach the claimed invention. Girasole never teaches or suggests, based on its finding on osteoclast, to administer IL-11 Ab (or any IL-11 antagonist) *in vivo* to treat the claimed pathological conditions. Were that be the case, an argument might be made that discovering the effect of IL-11 Ab on osteoblast merely explains the mechanism of the treatment. Such is clearly not the case here. Without the teaching of the instant invention about the effect of antagonizing IL-11 signaling in osteoblast, a skilled artisan simply cannot predict whether IL-11 Ab would be beneficial, ineffective, or detrimental to the subject pathological conditions.

The non-obviousness of the claimed invention is further supported by paragraph 13 of the Declaration, in which Applicants indicate that IL-11 Ab is effective *in vivo*, despite the cytokine redundancy issue well-known in the art at the time of filing the instant application. The paragraph listed numerous agents known to affect osteoclast function. In fact, even Girasole itself states that "IL-11 is unlikely to be the sole mediator of the osteoclastogenic effect." See page 1521, second column of Girasole. If IL-11 is not the sole mediator, a logical inference is that an antibody to IL-11 is unlikely to be therapeutically effective.

The overwhelming scientific evidence cited by Applicants above are not contradicted by any evidence that tend to support the Office Action position. Applicants respectfully remind the Examiner that the Supreme Court has articulated a standard whereby the PTO must establish a rational connection between the agency's fact-findings and its ultimate action. *Dickinson v. Zurko*, 119 S.Ct. 1816 (1999). In light of the inventor's Declaration and Applicants' arguments of record, and the presumption in favor of Applicants, it is respectfully asserted that the present rejection (agency adjudication) is not supported by "substantial evidence" from the Examiner, and as such, fails to rise above the "substantial evidence" test of Section 706(2)(D) of the Administrative Procedure Act (APA). At the minimum, the Office Action fails to pass the "arbitrary, capricious, an abuse of power, or otherwise not in accordance with law" standard set forth in Section

706(2)(A) of the APA. There is no reasonably acceptable logical reasoning based on sound fact finding, for the Office Action to insist that a skilled artisan would automatically believe an osteoclast inhibitor is *necessarily* an osteoblast stimulator, simply because these two cell types have opposite functions in controlling bone density.

Claim 50 is rejected under 35 U.S.C. 103 as being unpatentable over Girasole *et al.* (1995) in view of Kishimoto (supra) and Queen *et al.* (U.S. Pat. No. 5,530,101).

For the reasons above, the claimed invention is not obvious in view of Girasole and Kishimoto, and Queen does not in anyway correct these defects, even assuming a skilled artisan would be motivated to combine Queen with Girasole and Kishimoto to make humanized antibodies to inhibit osteoclast function.

Pursuant to MPEP 2143, "To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the reference themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations."

Therefore, none of the three requirement for establishing a *prima facie* case of obviousness is met. Reconsideration and withdrawal of rejection under 35 U.S.C. 103(a) are respectfully requested.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Should an extension of time be required, Applicants hereby petition for same and request that the extension fee and any other fee required for timely consideration of this submission be charged to **Deposit Account No. 18-1945.**

Respectfully Submitted,

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Skeletal Alterations in Ovariectomized Rats

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Summary. Female Sprague Dawley rats were subjected to either bilateral ovariectomy or sham surgery. Tetracycline derivatives were administered to each rat on two separate occasions to label sites of bone formation. All rats were sacrificed at 5 weeks postovariectomy and their proximal tibiae were processed undecalcified for quantitative bone histomorphometry. A twofold decrease in trabecular bone volume was noted in the proximal tibial metaphysis of ovariectomized rats. This bone loss was associated with elevated histomorphometric indices of bone resorption and formation. Ovariectomy increased osteoclast surface and numbers as well as osteoblast surface and numbers. Elevations in calcification rate and fractional trabecular bone surface with double tetracycline labels also suggest that bone formation was stimulated in ovariectomized rats. In addition, ovariectomized rats exhibited a greater rate of longitudinal bone growth relative to sham-operated control rats. These histomorphometric data indicate that ovariectomy induces marked bone loss and accelerated skeletal metabolism in rats.

Key words: Ovariectomy — Quantitative bone histomorphometry — Tetracycline — Osteoclasts — Osteoblasts.

Numerous studies utilizing biochemical and radiographic techniques have shown that bone loss occurs in ovariectomized rats [1–5]. However, this phenomenon is not well documented by histologic methods. Hodgkinson et al. [6] detected a reduced mass of trabecular bone in the caudal vertebrae of ovariectomized rats. These animals exhibited an increment in osteoid surface and a small, nonsignificant increase in resorption surface. Tetracycline-based analyses of skeletal alterations in ovariectomized rats are lacking. The purpose of the current study is to determine by histomorphometric methods the extent of trabecular bone loss and associated abnormalities in bone resorption and formation in ovariectomized rats.

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Materials and Methods

The experimental animals were 18 female Sprague Dawley rats that were approximately 75 days of age and weighed an average of 220 g. All rats were anesthetized with an i.p. injection of ketamine hydrochloride and xylazine at doses of 50 mg/kg body weight and 10 mg/kg body weight, respectively. Bilateral ovariectomies were performed in nine rats from a dorsal approach [7]. Nine control rats were subjected to sham surgeries in which the ovaries were exteriorized. Success of ovariectomy was confirmed at autopsy by failure to detect ovarian tissue and observation of marked atrophy of the uterine horn. After surgery, all rats were housed individually with food (Purina Rat Laboratory Chow) and water available ad libitum. Tetracycline derivatives were administered to each rat at a dose of 20 mg/kg body weight on two separate occasions preceding the day of sacrifice. Tetracycline chelates calcium and deposits primarily at sites of the initial calcification of new bone matrix [8,9]. Oxytetracycline hydrochloride (Pfizer Inc., Brooklyn, NY) was administered to each rat on the ninth day prior to sacrifice. After a 5-day period, during which no tetracycline was administered, all rats were injected i.p. with demeclocycline (Lederle Laboratories, Pearl River, NY) on the third day prior to sacrifice. This regimen results in deposition of a double tetracycline label at bone surfaces that are actively forming throughout the injection period.

At 5 weeks postovariectomy, all rats were sacrificed by cervical dislocation under ketamine anesthesia. The proximal tibiae were defleshed and placed in 10% phosphate-buffered formalin for 24 hours. The bone specimens were then dehydrated in ethanol and embedded undecalcified in methyl methacrylate [10]. Longitudinal sections of 4 μm thickness were cut with an AO Autocut/Jung 1150 microtome and stained according to Goldner's method [11]. Bone parameters were measured in these sections at a magnification of 400× with a Merz eyepiece reticle [12] consisting of 36 points and 6 semicircular lines within a

Table 1. Histomorphometric parameters in the proximal tibial metaphysis of ovariectomized and control rats

	Trabecular bone volume (%)	Osteoclast surface (%)	Osteoblast surface (%)	Osteoclasts/mm	Osteoblasts/mm
Ovariectomized (n = 9)	13.2[a] ±5.1	12.0[b] ±3.7	10.2[a] ±5.1	4.1[b] ±1.3	5.3[b] ±3.5
Control (n = 9)	25.9 ±4.4	7.4 ±1.8	2.8 ±1.4	2.7 ±0.5	1.7 ±0.8

All parameters for ovariectomized animals are different from control parameters at the levels of significance listed below
[a] $P < 0.001$
[b] $P < 0.01$

square. Two sections of the proximal tibial metaphysis, equivalent to 14 mm² of bone tissue, were sampled in each animal. This area was standardized in relation to the growth plate–metaphyseal junction.

The number of points superimposed over mineralized tissue (calcified cartilage and bone), osteoid (unmineralized bone matrix), and bone marrow were recorded. The fractional area of mineralized tissue, commonly referred to as trabecular bone volume, was determined by dividing the number of points lying over mineralized tissue by the total number of points. The occurrence of osteoid in both control and ovariectomized rats was minimal (<1%) and, therefore, could not be reliably quantified by the manual techniques employed in this study. The intersections of the semicircular reticle lines with the bone–bone marrow interface were classified as resting, osteoblast, or osteoclast surface. Resting surface is defined as trabecular bone surface without adjacent osteoblasts or osteoclasts. Osteoblast surface is defined as trabecular surface lined with osteoblasts, whereas irregular or scalloped trabecular surface with adjacent osteoclasts is classified as osteoclast surface. Osteoblast surface (%) was determined by dividing the number of intersects with bone surfaces lined by osteoblasts by the total number of intersects. Osteoclast surface (%) was calculated in a similar manner. The numbers of osteoblasts and osteoclasts adjacent to trabecular bone surfaces of the proximal tibial metaphysis were also quantified. These data are expressed as number of bone cells per millimeter trabecular bone perimeter. This latter parameter was determined by multiplying the total number of intersects by the grid constant, d, which is equal to the distance between grid points [12].

Tetracycline-based data were collected from unstained, 10-μm thick sections of the proximal tibial metaphysis. To measure the rate of longitudinal bone growth, the distance between the fluorescent tetracycline band that parallels the growth plate and the growth plate–metaphyseal junction was quantified with a calibrated eyepiece micrometer [13] at five equally-spaced sites per section. These measurements were performed under ultraviolet illumination at a magnification of 200× in two sections per animal. The rate of longitudinal bone growth was calculated by dividing the distance between the tetracycline band and the growth plate–metaphyseal junction by the time interval between administration of the tetracycline label and sacrifice.

Discrete tetracycline labels were present in lamellar bone of the secondary spongiosa. Intersects of semicircular grid lines with trabecular bone surfaces were categorized according to the presence or absence of tetracycline labels. Trabecular bone surfaces without tetracycline labels were considered to be nonforming. The fraction of actively forming trabecular surface was determined by dividing the number of intersects with double-labeled surface by the total number of intersects [14]. In addition, the distance between the two tetracycline markers that comprise a double label was meaured with a calibrated eyepiece micrometer at three or four equally spaced sites per double label. These measurements were performed on an average of 20 double tetracycline labels per animal. Calcification rate was calculated by dividing the interlabel distance by the time interval between administration of the two tetracycline markers. These values were not corrected for the random relation between the plane of section and the plane of tetracycline markers in trabecular bone [14].

Data are expressed as the mean ± SD of the control and ovariectomized groups. Statistical differences between the two groups were evaluated with the two-tailed Student's t test. P values of less than 0.05 were considered to be significant.

Results

Ovariectomized rats weighed significantly more than control rats. The mean body weight of nine ovariectomized rats was 298.4 ± 26.3 g, while the nine control rats weighed an average of 253.3 ± 14.0 g. This difference is highly significant at the level of $P < 0.001$.

Table 1 lists values for static histomorphometric parameters in the proximal tibial metaphysis of ovariectomized and control rats. Trabecular bone mass declined by a factor of 2 in ovariectomized rats ($P < 0.001$). This marked bone loss at 5 weeks postovariectomy can be visualized in Fig. 1. Ovariectomized rats also exhibited histologic evidence for enhanced bone resorption and formation. Osteoclast surface and numbers were significantly elevated ($P < 0.01$) in ovariectomized rats relative to control rats. Ovariectomized rats were also characterized by significant increments in osteoblast surface ($P < 0.001$) and numbers ($P < 0.01$).

Tetracycline-based data from the proximal tibial metaphysis are listed in Table 2. An increased rate of longitudinal bone growth was observed in ovariectomized rats ($P < 0.001$). In addition, these animals had significant elevations in actively forming

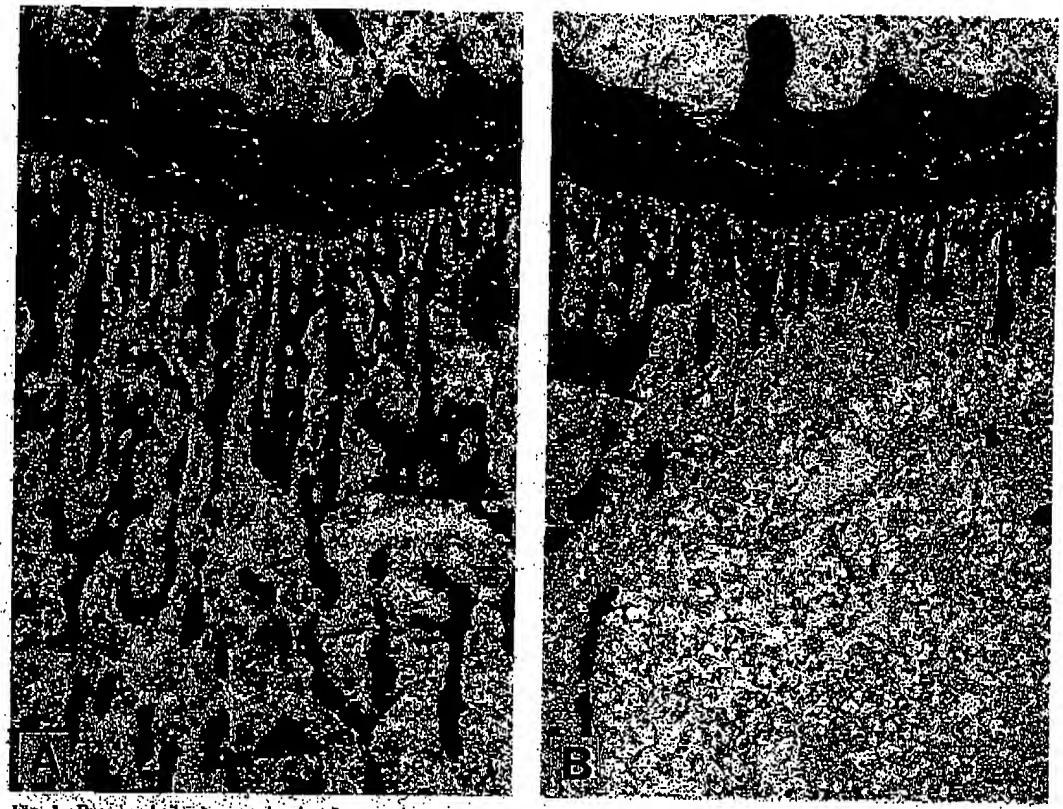

Fig. 1. Proximal tibial metaphysis of control (A) and ovariectomized (B) rats. Note the reduced mass of darkly stained trabecular bone in the ovariectomized animal. (Von Kossa stain, ×25).

Table 2. Tetracycline-based parameters in the proximal tibial metaphysis of ovariectomized and control rats

	Longitudinal bone growth (μm/day)	Forming bone surface (%)[a]	Calcification rate (μm/day)
Ovariectomized (n = 7)	36.5[b] ±6.0	12.0[d] ±10.7	1.6[c] ±0.4
Control (n = 8)	26.7 ±3.1	2.9 ±1.7	1.2 ±0.2

[a] Forming bone surface is defined as bone surface with a double tetracycline label
[b] $P < 0.001$
[c] $P < 0.025$
[d] $P < 0.05$

trabecular bone surface ($P < 0.05$) and calcification rate ($P < 0.025$).

Discussion

This study demonstrates that rapid bone loss occurs in the proximal tibial metaphysis of ovariectomized rats. A twofold decrease in trabecular bone volume was noted at 5 weeks postovariectomy. In addition, osteoclastic and osteoblastic data suggest that ovariectomy stimulates bone resorption and formation. The observed increases in actively forming trabecular bone surface and calcification rate, as determined by double tetracycline labeling, also indicate that bone formation was elevated in ovariectomized rats. Alterations in bone formation in response to ovariectomy appear to be somewhat variable, as indicated by relatively high standard deviations for osteoblastic and tetracycline-based parameters. In view of the observed increases in both bone resorption and formation, the loss of trabecular bone mass associated with ovariectomy is unexplained. The pathogenetic mechanism may involve a greater increment in bone resorption relative to the increment in bone formation so that net loss of skeletal mass occurs. Such a mechanism has been proposed for the development of postmenopausal bone loss in humans [15]. Although surface-based and cellular parameters suggest that bone for-

mation was increased to a greater extent than bone resorption in ovariectomized rats, these static measurements are not indicative of osteoclastic activity. An accelerated rate of bone resorption by individual osteoclasts as well as an increased osteoclast population may result in bone loss in ovariectomized rats even in the presence of increased osteoblast numbers and activity. It is also important to note that 5 weeks postovariectomy may not be of sufficient duration to achieve a steady state in the skeletal response to estrogen deficiency.

Ovariectomized rats exhibit an increased rate of weight gain. This phenomenon has been reported by other investigators [1–4]. In contrast to the protective effect of obesity against osteoporosis in postmenopausal women [16–18], marked bone loss occurred in obese ovariectomized rats. The effect of obesity on histologic indices of bone resorption and formation has not been documented. Nevertheless, the possibility that increased body mass affects bone turnover in ovariectomized rats cannot be ruled out.

Estrogen is thought to act as an antagonist to parathyroid hormone (PTH) [19]. The loss of this antagonism after ovariectomy may result in increased skeletal sensitivity to PTH. Our histologic data are consistent with this theory. The osteoclast and osteoblast populations in the long bone metaphysis of rats are known to increase in response to PTH [20,21]. Physiologic doses of PTH induce a coupled increase in bone resorption and formation in young adult dogs [22]. Tam et al. [23] found that rats infused with PTH had elevated calcification rates. The skeletal characteristics of ovariectomized rats appear to be similar to those of rats treated with PTH.

Estrogen is also thought to act as an antagonist to growth hormone [24]. Widening of tibial epiphyseal cartilage was inhibited by estrogen in hypophysectomized rats treated with growth hormone [25]. Whitson et al. [26] demonstrated that the most rapid phase of longitudinal bone growth in rats occurs when serum estrogen levels are minimal. Our finding of an accelerated rate of longitudinal bone growth in ovariectomized rats may be due to loss of estrogenic antagonism to the stimulatory effects of growth hormone.

Although rats have a juvenile skeleton in which modeling predominates, it is interesting to compare histomorphometric data from ovariectomized rats to that of postmenopausal patients with adult, remodeling bone. Increased resorption surfaces were detected in the iliac crest of postmenopausal women by microradiography [27] and histologic techniques [28]. On the other hand, tetracycline-based histomorphometric analyses indicated that the majority of osteoporotic patients had relatively normal indices of bone resorption and formation [29,30]. However, several subgroups were identified in which osteoporotic patients exhibited high levels of bone remodeling or a pathologically low rate of bone formation. These heterogeneous findings may reflect different phases of the disease process. Parfitt et al. [31] hypothesized that the initial rapid phase of postmenopausal bone loss is associated with an increased rate of bone remodeling and enhanced osteoclastic activity. With time, bone remodeling is thought to diminish, but loss of skeletal mass continues at a slower rate, probably due to depressed bone formation. Calcium kinetic and biochemical data support the contention that an increased rate of bone remodeling occurs in women soon after naturally occurring or surgical menopause [15,32]. Our histomorphometric data indicate that accelerated bone metabolism also occurs in rats at early times postovariectomy.

In summary, the current study demonstrates that ovariectomy induces marked loss of trabecular bone in the proximal tibial metaphysis of rats. The observed bone loss is associated with an increased rate of longitudinal bone growth and elevated histomorphometric indices of bone resorption and formation.

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OVX ↑ both BF & BR

Temporal Relationship between Bone Loss and Increased Bone Turnover in Ovariectomized Rats

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Summary. To characterize osteopenic changes in ovariectomized (OVX) rats as a function of time, female Sprague Dawley rats (240 g body weight, 90 days old) were subjected to bilateral ovariectomy or sham surgery and killed at various times from 14–180 days postovariectomy. The proximal tibial metaphysis was processed undecalcified for quantitative bone histomorphometry. Osteopenia and increased indices of bone resorption and formation were detected in OVX rats as early as 14 days. Longitudinal bone growth was also significantly increased by ovariectomy at 14 days, but returned to control levels at all later times. In OVX rats, osteopenia became progressively more pronounced with time up to 100 days postovariectomy, after which trabecular bone volume appeared to stabilize at the markedly reduced level of 5%. Changes in osteoclast surface, osteoblast surface, and fluorochrome-based indices of bone formation in OVX rats followed a similar time course. The maximal increase in these parameters occurred during the first several months postovariectomy followed by a gradual decline toward control levels. Our results indicate that the initial rapid phase of bone loss in OVX rats is coincident with the maximal increase in bone turnover. At later times postovariectomy, bone loss and bone turnover both subside. These findings emphasize the close temporal association between the development of osteopenia and increased bone turnover in OVX rats.

Key words: Ovariectomy — Osteopenia — Bone turnover — Time course — Quantitative bone histomorphometry.

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Osteopenia has been a consistent finding in ovariectomized rats [1–9]. Histomorphometric studies in our laboratory have shown that this phenomenon is associated with increased bone turnover [7–9]. For the most part, previous studies have been performed at a single time point during the early stages of estrogen deficiency; therefore, it is not known with certainty whether the observed bone loss and increased bone turnover continue unabated with time. The purpose of the current study is to characterize osteopenic changes in ovariectomized rats as a function of time from 14–180 days postovariectomy.

Materials and Methods

The experimental animals were 168 female Sprague Dawley rats, (Charles River Laboratory Inc., Wilmington, MA) 90 days old and weighing an average of 240 g at the beginning of the study. All rats were anesthetized with an i.p. injection of ketamine hydrochloride and xylazine at doses of 50 mg/kg body weight and 10 mg/kg body weight, respectively. Bilateral ovariectomies (OVX) were performed in half of the rats from a dorsal approach [10]. The remainder were subjected to sham surgeries. All rats were housed individually at 25°C with a 13 h/11 h light/dark cycle. Food (Purina Rat Laboratory Chow, St. Louis, MO) was available *ad libitum* to the sham-operated control rats. To minimize the increase in body weight associated with ovariectomy [9], the food consumption of OVX rats was restricted to that of the control rats (pair-feeding). Demeclocycline (Lederle Laboratories, Pearl River, NY) and calcein (Sigma Co., St. Louis, MO) were administered to each rat by i.p. injection at a dose of 10 mg/kg body weight on the 12th and 5th days before death, respectively. This regimen resulted in deposition of a double fluorochrome label at bone surfaces that were actively mineralizing throughout the injection period.

Twelve control and 12 OVX rats were killed by exsanguination under ketamine/xylazine anesthesia at 14, 35, 52, 70, 100, 125, and 180 days postovariectomy. In addition, a group of eight baseline control rats was sacrificed on day 0. Success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine

horns. The proximal tibiae were defleshed and placed in 10% phosphate-buffered formalin for 24 hours. The bone specimens were then dehydrated in ethanol and embedded undecalcified in methyl methacrylate [11]. Longitudinal sections (4 μm thick) were cut with an AO Autocut/Jung 1150 microtome and stained with a modified Masson-Goldner trichrome [11]. Bone parameters were measured in the proximal tibial metaphysis with the Bioquant Bone Morphometry Package (R&M Biometrics Corp., Nashville, Tenn.). Trabecular areas and lengths were traced with a cursor and Hipad digitizing tablet adjacent to a Nikon Labophot microscope. The light within the cursor can be visualized in the microscopic field when used in conjunction with a camera lucida. Raw data were stored in an Apple IIe microcomputer interfaced to the digitizing tablet. Values for bone histomorphometric parameters were then calculated with Bioquant software. Trabecular bone volume (%) and osteoclast and osteoblast surface as percentages of total trabecular surface length were measured in this manner. Bone parameters were quantified in trabecular bone tissue at distances greater than 1 mm from the growth plate-metaphyseal junction to exclude the primary spongiosa. Additional details of the sample site and data calculations have been published elsewhere [7, 8]. In general, two sections of the proximal tibia with ~40 mm of trabecular perimeter were sampled in each control animal. The relative lack of bone spicules in OVX rats, especially at later times postovariectomy, made it necessary to sample additional sections in these animals. Therefore, surface-based parameters were measured in three or more sections in each OVX rat to approximate the trabecular perimeter sampled in control rats.

Fluorochrome-based parameters were measured in unstained, 10 μm thick sections of the proximal tibial metaphysis. The rate of longitudinal bone growth, percentage of trabecular bone surface with a double fluorochrome label (active formation surface), and calcification rate were measured with the Bioquant system described above. In addition, bone formation rate (tissue level, total surface referent) was calculated by multiplying active formation surface by calcification rate [12]. Values for calcification rate were not corrected for obliquity of the plane of section in trabecular bone [12].

Data are expressed as the mean ± SD of the control and OVX groups at each time point. Statistical differences between the two groups were evaluated with the two-tailed Student's *t* test. *P* values of less than 0.05 were considered significant.

Results

Despite pair-feeding, OVX rats weighed significantly more than control rats at all times later than 14 days postovariectomy (data not shown). The final body weights of OVX and control rats at 180 days were 409.0 ± 29.0 g and 346.7 ± 26.5 g, respectively ($P < 0.001$).

Bone histomorphometric parameters in the proximal tibial metaphysis are plotted as a function of time postovariectomy in Figures 1 and 2. Trabecular bone volume (Fig. 1A) remained relatively constant in control rats (~30%) for the duration of the experiment. Osteopenia was detected in OVX rats as early as 14 days and became more pronounced with time up to 100 days postovariectomy. At later times, trabecular bone volume appeared to be maintained in OVX rats at a markedly reduced level of ~ 5% ($P < 0.001$).

OVX rats exhibited a transient increase in longitudinal bone growth (Fig. 1B). This parameter was significantly increased by ovariectomy only at 14 days, then was nearly identical in OVX and control rats at all later times.

Figures 1C and 1D depict values for osteoclast and osteoblast surface, respectively. Ovariectomy induced a rapid increase in both osteoclast and osteoblast surface as early as 14 days. These parameters remained elevated in OVX rats up to 100 days. At later times, OVX rats exhibited trends for increased osteoclast and osteoblast surface, but statistical significance was not observed.

Fluorochrome-based parameters are depicted in Figures 1E and 1F. Active formation surface was significantly increased in OVX rats at all times from 14–180 days postovariectomy (Fig. 1E). The maximal increase in this parameter occurred during the first month, followed by a gradual decline toward control levels. Calcification rate (Fig. 1F) was nearly identical in control and OVX rats at 14 days, but significant increases were observed in OVX rats from 35–100 days postovariectomy. Afterwards, calcification rate was not significantly different in the two groups.

OVX rats were characterized by an increased bone formation rate throughout the course of the experiment (Fig. 2). This fluorochrome-based parameter is perhaps the most meaningful index of bone turnover. Bone formation rate was increased maximally at the end of the first month postovariectomy and remained highly elevated up to 100 days. Although still elevated, bone formation rate in OVX rats declined toward control levels at later times postovariectomy.

Discussion

It is now well established that increased bone turnover occurs during the early stages of estrogen deficiency. This phenomenon has been previously observed in rats [7–9], dogs [13], baboons [14], and humans [15]. However, a serial histomorphometric study of bone changes after loss of ovarian function has never been reported. The current study demonstrates that the initial rapid phase of bone loss in the proximal tibial metaphysis of OVX rats is coincident with the maximal increase in bone turnover. Osteopenia was detected in OVX rats as early as 14 days postovariectomy and became progressively more pronounced up to 100 days; afterwards, tibial

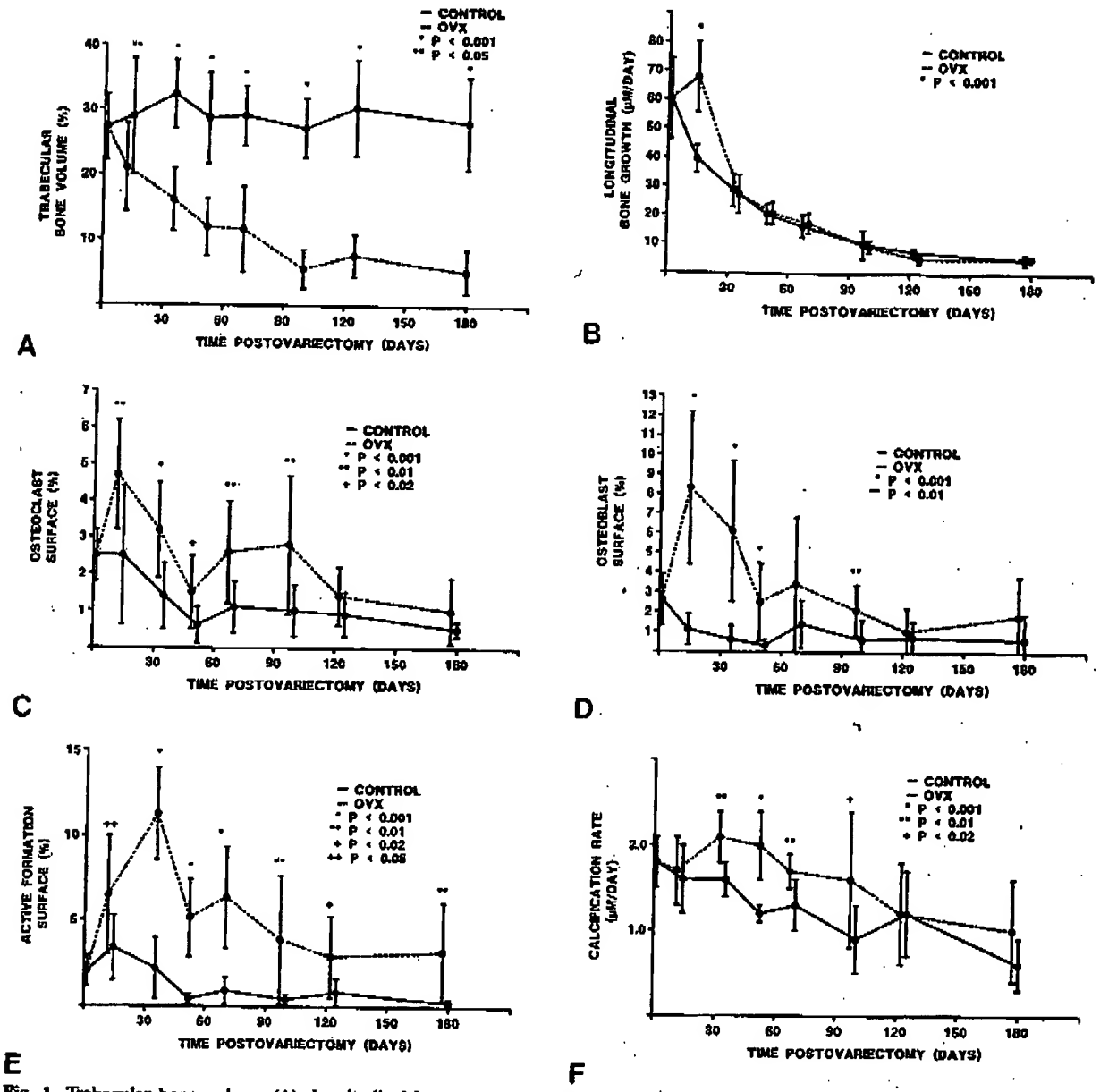

Fig. 1. Trabecular bone volume (A), longitudinal bone growth (B), osteoclast surface (C), osteoblast surface (D), active formation surface (E), and calcification rate (F) in the proximal tibial metaphysis are plotted as a function of time postovariectomy. Each data point for the control (solid lines) and OVX (broken lines) groups is the mean ± SD of 12 animals. The data point for the baseline control group (day 0) is the mean ± SD of eight animals. Levels of significance were determined with the two-tailed Student's *t* test.

trabecular bone volume appeared to stabilize in OVX rats. Histologic indices of bone resorption and formation follow a similar time course. These indices were found to be elevated maximally in OVX rats during the first month postovariectomy followed by a gradual decline toward control levels. At later times postovariectomy, during which bone loss diminished in OVX rats, histologic indices of bone resorption and formation were only marginally elevated. We interpret these data as indicative of a close temporal association between the initial development of osteopenia in OVX rats and a marked increase in bone turnover. Despite the relatively short life span of rodents, these findings are

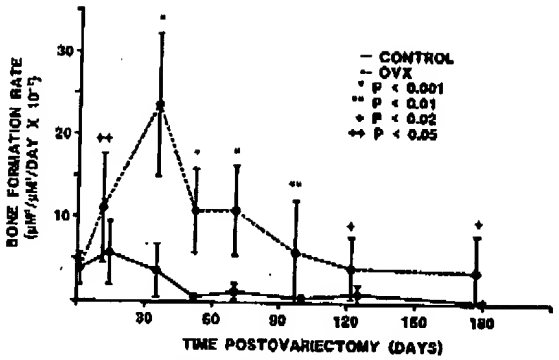

Fig. 2. Bone formation rate (tissue level, total surface referent) in the proximal tibial metaphysis of control (solid line) and OVX (broken line) groups is plotted as a function of time postovariectomy. See Figure 1 legend for details.

consistent with current concepts of skeletal dynamics in estrogen-deficient women [16–18]. Although rates of bone loss are variable in postmenopausal women, a certain subgroup exhibits a rapid phase of bone loss during the early postmenopausal period [17]. The calcium kinetic studies of Heaney et al. [15] first indicated that increased bone turnover occurs at this time. Numerous reports of elevations in biochemical indices of bone turnover during the early stages of estrogen deficiency in women [19–22] have since confirmed this finding. With time after menopause, the rates of bone loss and bone turnover both diminish [16–18]. Therefore, the temporal association between rapid bone loss and maximally increased bone turnover is qualitatively similar in estrogen-deficient rats and humans.

Based on cellular parameters, one might surmise that bone formation is increased to a greater extent than bone resorption in OVX rats. For example, an eightfold increase in osteoblast surface was observed in OVX rats at 14 days postovariectomy compared with a twofold increase in osteoclast surface. However, it is important to note that these static parameters are not indicative of cellular activity and corresponding bone formation and resorption rates. Dynamic fluorochrome-based parameters provide strong evidence for an accelerated bone formation rate in OVX rats. Unfortunately, bone resorption rate cannot be measured directly by histomorphometric techniques. Nevertheless, the observation of marked bone loss in OVX rats despite increased bone formation indicates that the increment in bone resorption exceeds the increment in bone formation. It follows that osteoclastic activity must be markedly increased in OVX rats. Along similar lines, Gruber et al. [23] recently reported that individual osteoclast activity is significantly greater than osteoblast activity in postmenopausal patients.

At later times postovariectomy, osteoblast surface in OVX rats declined to control levels whereas active formation surface (double-labeled surface) remained significantly increased. This apparent discrepancy may be explained by age-related changes in osteoblast morphology. In contrast to the plump, cuboidal osteoblasts of young rats, these bone-forming cells become more flattened in appearance in older rats. Consequently, osteoblasts are more difficult to identify in the older animals. This probably resulted in an underestimate of osteoblast surface at later times postovariectomy. In any case, dynamic fluorochrome-based parameters are more meaningful than static cellular parameters as indices of bone formation. The former parameters indicate that bone formation rate is still significantly increased in OVX rats at later times postovariectomy.

One must exercise caution in comparing data between the juvenile rat skeleton and adult human bone due to the occurrence of modeling and longitudinal bone growth in rodents. Since recent evidence suggests that at least some remodeling occurs in rat trabecular bone [24], the skeletal dynamics of these animals may be more relevant to the adult human situation than previously thought. In any case, estrogen deficiency stimulates bone turnover in both modeling and remodeling skeletons. Alterations in longitudinal bone growth in OVX rats could conceivably affect trabecular bone volume in the long bone metaphysis. However, ovariectomy increased longitudinal bone growth only during the first several weeks after surgery. By 35 days postovariectomy and at all later times, longitudinal bone growth was nearly identical in control and OVX rats. Because considerable bone loss occurred in OVX rats between 35 and 100 days postovariectomy, it is clear that alterations in longitudinal bone growth do not play a major role in the development of osteopenia in OVX rats.

In summary, the current study demonstrates that the initial rapid phase of bone loss in OVX rats is coincident with the maximal increase in bone turnover. At later times postovariectomy, trabecular bone mass appears to stabilize as bone turnover declines toward control levels. These findings emphasize the close temporal relationship between increased bone turnover and the development of osteopenia in OVX rats.

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Histologic Evidence for Osteopenia and Increased Bone Turnover in Ovariectomized Rats

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Abstract

Bone histomorphometry was performed in the proximal tibia and lumbar vertebra of female Sprague-Dawley rats subjected to either bilateral ovariectomy or sham surgery. Ovariectomized rats killed 10 weeks after surgery were characterized by osteopenia and histomorphometric indices of increased bone turnover at both skeletal sites. Osteoclast surface and number as well as osteoblast surface and number were significantly increased in ovariectomized rats. Tetracycline-based data were consistent with accelerated bone formation in response to ovariectomy. Active formation surface (double-labeled surface), calcification rate, and bone formation rate (tissue level, total surface referent) were all significantly elevated in the proximal tibia of ovariectomized rats. Ovariectomy also increased active formation surface and bone formation rate in the lumbar vertebra, but the vertebral calcification rate was nearly identical in control and ovariectomized rats. Osteopenia and dynamic tetracycline-based estimates of increased bone turnover in ovariectomized animals were more pronounced in the proximal tibia relative to the lumbar vertebra. Longitudinal bone growth in the proximal tibia and mineralization lag time at the tibial cortical endosteal surface were not affected by ovariectomy. These histomorphometric data indicate that ovariectomy induces osteopenia and accelerated skeletal metabolism in rats.

Key Words: Ovariectomy–Bone Histomorphometry–Tetracycline–Osteoclasts–Osteoblasts–Bone Turnover.

Introduction

Ovariectomy is known to induce osteopenia in rats (Saville, 1969; Aitken et al., 1972; Lindgren and Lindholm, 1979; Lindgren and DeLuca, 1982; Beall et al., 1984). However, the osteopenic changes have not been studied extensively by histologic methods. Hodgkinson et al. (1978) detected an increment in osteoid surface and a nonsignificant increase in resorption surface in the caudal vertebrae of ovariectomized rats. It was recently reported that marked bone loss occurs in the proximal tibial metaphysis of ovariectomized rats as early as 5 weeks postovariectomy (Wronski et al., 1985). This bone loss was associated with an increased rate of longitudinal bone growth and elevated histomorphometric indices of bone resorption and formation. In this communication, we extend our observations in ovariectomized rats to include histomorphometric studies of the proximal tibia and lumbar vertebra at 10 weeks postovariectomy.

Materials and Methods

Female Sprague-Dawley rats (approximately 75 days old and weighing an average of 220 g at the beginning of the study) were used. All rats were anesthetized with IP ketamine hydrochloride and xylazine at doses of 50 mg/kg body weight and 10 mg/kg body weight, respectively. Bilateral ovariectomies were performed in 13 rats from a dorsal approach (Waynforth, 1980). Eleven control rats were subjected to sham surgeries in which the ovaries were exteriorized. Success of ovariectomy was confirmed at autopsy by failure to detect ovarian tissue and observation of marked atrophy of the uterine horn. After surgery, all rats were housed individually, with food (Purina Rodent Laboratory Chow, St. Louis, MO) and water available ad libitum. Tetracycline derivatives were administered to each rat at a dose of 20 mg/kg body weight on two separate occasions. Oxytetracycline hydrochloride (Pfizer Inc., Brooklyn, NY) was injected IP on the ninth day before killing. After a 5-day period during which no tetracycline was administered, all rats were injected IP with demeclocycline (Lederle Laboratories, Pearl River, NY) on the third day before killing. This regimen resulted in deposition of a double tetracycline label at bone surfaces that were actively mineralizing throughout the injection period.

All rats were killed at 10 weeks postovariectomy by cervical dislocation under ketamine anesthesia. Terminal blood samples were collected from the abdominal aorta of 6 control and 6 ovariectomized rats. Serum calcium was measured spectrophotometrically by the cresolphthalein/diethylamine colorimetric method. The proximal tibiae and lumbar vertebrae 1 and 2 were defleshed and placed in 10% phosphate-buffered formalin for 24 h. The bone specimens were then dehydrated in ethanol and embedded undecalcified in methylmethacrylate (Baron et al., 1983). Longitudinal sections (4 μm) were cut with an AO Autocut/Jung 1150 microtome

and stained with Masson-Goldner trichrome. The following bone parameters were measured manually at a magnification of ×400 with the aid of a Merz eyepiece reticle (Merz and Schenk, 1970): trabecular bone volume (%), osteoclast surface (%), osteoblast surface (%), and numbers of osteoclasts and osteoblasts per mm trabecular bone perimeter (Wronski et al., 1985). Bone parameters were quantified within a 2 × 3 mm area of trabecular bone tissue that was standardized at distances greater than 1 mm from the growth plate–metaphyseal junction to exclude the primary spongiosa. Two sections each of the proximal tibia and lumbar vertebrae were analyzed in each animal. The total area of trabecular bone tissue sampled at each skeletal site was ≈ 13 mm².

Tetracycline-based data were collected from unstained, 10 μm sections of the proximal tibial metaphysis and lumbar vertebrae 1 and 2. The rate of longitudinal bone growth, percentage of trabecular bone surface with a double tetracycline label (active formation surface), and calcification rate were measured with a Merz eyepiece reticle and calibrated eyepiece micrometer (Wronski et al., 1985). Values for calcification rate were not corrected for obliquity of the plane of section in trabecular bone. In addition, the bone formation rate (tissue level, total surface referent) was calculated by multiplying the active formation surface by the calcification rate (Frost, 1983a). Since trabecular bone surfaces with single tetracycline labels were not included in this calculation, the derived values for bone formation rate probably underestimate the actual bone formation rate (Frost, 1983b).

The occurrence of osteoid in both control and ovariectomized rats was minimal (< 1%). Therefore, osteoid parameters could not be reliably quantified in trabecular bone tissue by the manual techniques employed in this study. However, a prominent osteoid seam was present along the cortical endosteal surface of the proximal tibia. Osteoid seam width was measured at 10 sites per section with a calibrated eyepiece micrometer at a magnification of ×400. Two sections of the proximal tibia were sampled in each animal. The distance between the two tetracycline labels adjacent to the cortical endosteal surface was measured in a similar manner. Calcification rate was determined by dividing this distance by the time interval between administration of the tetracycline markers. Mineralization lag time of the cortical endosteal surface of the proximal tibia was calculated by dividing osteoid seam width by calcification rate (Baron et al., 1984).

Data are expressed as the mean ± SD of control and ovariectomized groups. Statistical differences between the two groups were evaluated with the two-tailed Student's *t*-test. *P* values of less than 0.05 were considered to be significant.

Results

The mean body weights of ovariectomized and control rats were 329.5 ± 16.0 g and 272.4 ± 13.9 g, respectively. This difference is highly significant at the level of $P < 0.001$. The mean serum calcium was 10.7 mg/dl in both control and ovariectomized rats.

Values for static histomorphometric parameters in the proximal tibia and lumbar vertebra are listed in **Table I**. Ovariectomized rats were characterized by reduced trabecular bone volume and elevated indices of bone resorption and formation at both skeletal sites. Statistically significant increments in osteoclast surface and number as well as osteoblast surface and number were noted in ovariectomized rats. Trabecular bone loss in these animals was more pronounced in the proximal tibia relative to the lumbar vertebra.

Tetracycline-based data are listed in **Table II**. The rate of longitudinal bone growth in the proximal tibia was nearly identical in ovariectomized and control rats. Longitudinal bone growth in the lumbar vertebra of all rats was too slow to be measured with the tetracycline regimen employed in this study. Active formation surface, calcification rate, and bone formation rate were all significantly elevated in the proximal tibia of ovariectomized rats. These animals also exhibited significant increments in active formation surface and bone formation rate in the lumbar vertebra. However, the vertebral calcification rate was approximately equal in control and ovariectomized rats. The difference in active formation surface between the two groups can be seen in **Figure 1**. Bone formation rate in ovariectomized rats was increased to a greater extent in the proximal tibia relative to the lumbar vertebra.

Histomorphometric parameters at the cortical endosteal surface of the proximal tibia are presented in **Table III**. Although osteoid seam width and calcification rate appear to be somewhat elevated in ovariectomized rats, these parameters are not significantly different from control values. Mineralization lag time was 1.4 days for both control and ovariectomized animals.

Discussion

This study demonstrates that ovariectomy induces osteopenia in the proximal tibia and lumbar vertebra of rats. Elevations in various histomorphometric indices of bone resorption and formation are suggestive of accelerated bone turnover at both skeletal sites. Static surface-based parameters indicate that bone formation was increased to a greater extent than bone resorption in ovariectomized rats. Although tetracycline-based data are consistent with enhanced osteoblastic activity in these animals, the life span of osteob-

Table I. Static histomorphometric parameters in the proximal tibia and lumbar vertebra of ovariectomized and control rats.

	Trabecular bone volume (%)	Osteoclast surface (%)	Osteoblast surface (%)	Osteoclasts/mm	Osteoblasts/mm
Tibia					
Ovariectomized ($n=13$)	10.5[a] ±3.5	15.5[b] ±4.9	5.7[a] ±3.8	3.0[b] ±0.9	3.4[a] ±2.2
Control ($n=11$)	25.9 ±4.8	10.8 ±3.8	0.8 ±0.7	2.2 ±0.8	0.6 ±0.6
Lumbar vertebra					
Ovariectomized ($n=13$)	28.4[b] ±3.7	9.7[a] ±2.9	3.5[b] ±1.8	2.0[a] ±0.5	2.1[b] ±1.0
Control ($n=11$)	32.6 ±4.7	4.9 ±1.6	1.2 ±1.1	1.0 ±0.4	0.8 ±0.7

[a] $P < 0.001$.
[b] $P < 0.05$.
[c] $P < 0.01$.

Table II. Tetracycline-based parameters in the proximal tibia and lumbar vertebra of ovariectomized and control rats.

	Longitudinal bone growth (μm/day)	Active formation surface (%)[a]	Calcification rate (μm/day)	Bone formation rate (μm³/μm² per day)
Tibia				
Ovariectomized	16.1	16.1[b]	1.7[b]	0.264[c]
(n=13)	±3.8	±10.0	±0.4	±0.162
Control	15.4	3.2	1.1	0.034
(n=9)	±2.6	±2.9	±0.5	±0.029
Lumbar vertebra				
Ovariectomized	—[d]	8.2[b]	1.2	0.101[b]
(n=13)		±4.0	±0.2	±0.053
Control	—	3.2	1.1	0.036
(n=9)		±1.2	±0.1	±0.014

[a]Defined as trabecular bone surface with a double tetracycline label.
[b]$P < 0.01$.
[c]$P < 0.001$.
[d]The rate of longitudinal bone growth in the lumbar vertebra is too low to be measured with the tetracycline regimen employed in this study.

lasts was not evaluated. If estrogen deficiency reduced osteoblastic longevity, the increment in bone formation in ovariectomized rats may not be as great as the cellular and tetracycline-based data indicate. The effect of ovariectomy on bone resorption rate and osteoclastic activity cannot be determined directly with available histomorphometric methods. Therefore, the mechanism for the decline in trabecular bone mass in ovariectomized rats in unclear. One possibility is that the increment in bone resorption and osteoclastic activity exceeded the increment in bone formation and osteoblastic activity so that net loss of bone occurred. Based on calcium kinetic analyses, Heaney et al. (1978) proposed such a mechanism for the development of osteopenia in women during the early postmenopausal period. It is also possible that the increased rate of longitudinal bone growth noted at earlier times postovariectomy (Wronski et al., 1985) may have contributed to marked osteopenia in the proximal tibia of ovariectomized rats.

Osteopenia in ovariectomized rats was more pronounced in the proximal tibia relative to the lumbar vertebra. This finding may be the result of a greater increase in bone turnover at the former skeletal site. Perhaps the most meaningful index of bone turnover is the bone formation rate derived from dynamic tetracycline-based data. Ovariectomized rats exhibited nearly an eightfold increase in bone formation rate in the tibia compared to a threefold increase in the lumbar vertebra (Table II). These data suggest that osteopenia in ovariectomized rats is more pronounced at skeletal sites with a greater relative increase in bone turnover. As mentioned, the increased rate of longitudinal bone growth detected at earlier times postovariectomy in the proximal tibia may also play a role in the development of more marked osteopenia in the tibia relative to the lumbar vertebra. Longitudinal bone growth is much slower at the latter skeletal site (Table II).

Out results should not be interpreted as providing evidence that trabecular bone loss during estrogen deficiency occurs primarily in the appendicular skeleton of adult mammals and humans. The tibia of growing rats has red (hematopoietic) marrow, whereas the distal long bones of skeletally mature mammals have yellow (fatty) marrow. Skeletal sites with red marrow are known to have greater rates of trabecular bone turnover than skeletal sites with yellow marrow (Wronski et al., 1980, 1981). Therefore, the marked osteopenia and accelerated bone turnover observed in the proximal tibia of growing ovariectomized rats may overestimate the magnitude of bone changes in the adult appendicular skeleton. On this basis, the lumbar vertebra, which retains the majority of its red marrow with increasing age in rats, other mammals, and humans, may be a superior sampling site for bone histomorphometric studies. Furthermore, Baron et al. (1984) reported that skeletal processes in the vertebral column of growing rats are similar to the remodeling activity of adult bone.

We previously described osteopenia and elevated histomorphometric indices of bone resorption and formation in the proximal tibia of rats at 5 weeks postovariectomy (Wronski et al., 1985). In the current study nearly identical bone changes were detected at the same sample site in the proximal tibial metaphysis at 10 weeks postovariectomy. In addition, histomorphometric analysis of the lumbar vertebra at 10 weeks postovariectomy revealed osteopenia and a qualitatively similar increase in bone turnover. It is unclear whether these time periods are of sufficient duration to achieve a steady state in the skeletal response to estrogen deficiency. Baron et al. (1984) reported that the duration of the remodeling sequence (sigma) in the caudal vertebrae of growing rats is 35–40 days. If it can be assumed that sigma is of a similar duration in other parts of the rat vertebral column, the increased bone turnover detected in the lumbar vertebrae of rats at 10 weeks postovariectomy may be a long-term rather than a transient effect. However, the relative increase in osteoblastic parameters appears to be greater than the increase in osteoclastic parameters in these animals. This finding suggests that ovariectomized rats may not yet have achieved a steady state with respect to the skeletal effects of estrogen deficiency at 10 weeks postovariectomy. Histomorphometric measurements at multiple time periods postovariectomy are needed to resolve this issue. On the other hand, the increased rate of longitudinal bone growth previously detected in the proximal tibia at 5 weeks postovariectomy (Wronski et al., 1985) is apparently transient. This parameter was found to be normal in ovariectomized rats at 10 weeks postovariectomy.

Detailed histomorphometric studies have not been performed in women specifically during the early stages of estrogen deficiency. However, calcium kinetic data indicate that increments in both bone resorption and formation occur during the first 5 years of menopause (Heaney et al., 1978). Furthermore, various biochemical indices of bone turnover, such as serum osteocalcin (bone GLA protein), alkaline phosphatase, and urinary hydroxyproline/creatinine, have been shown to be elevated soon after menopause (Christiansen et al., 1982; Gennari et al., 1984; Riis et al., 1984).

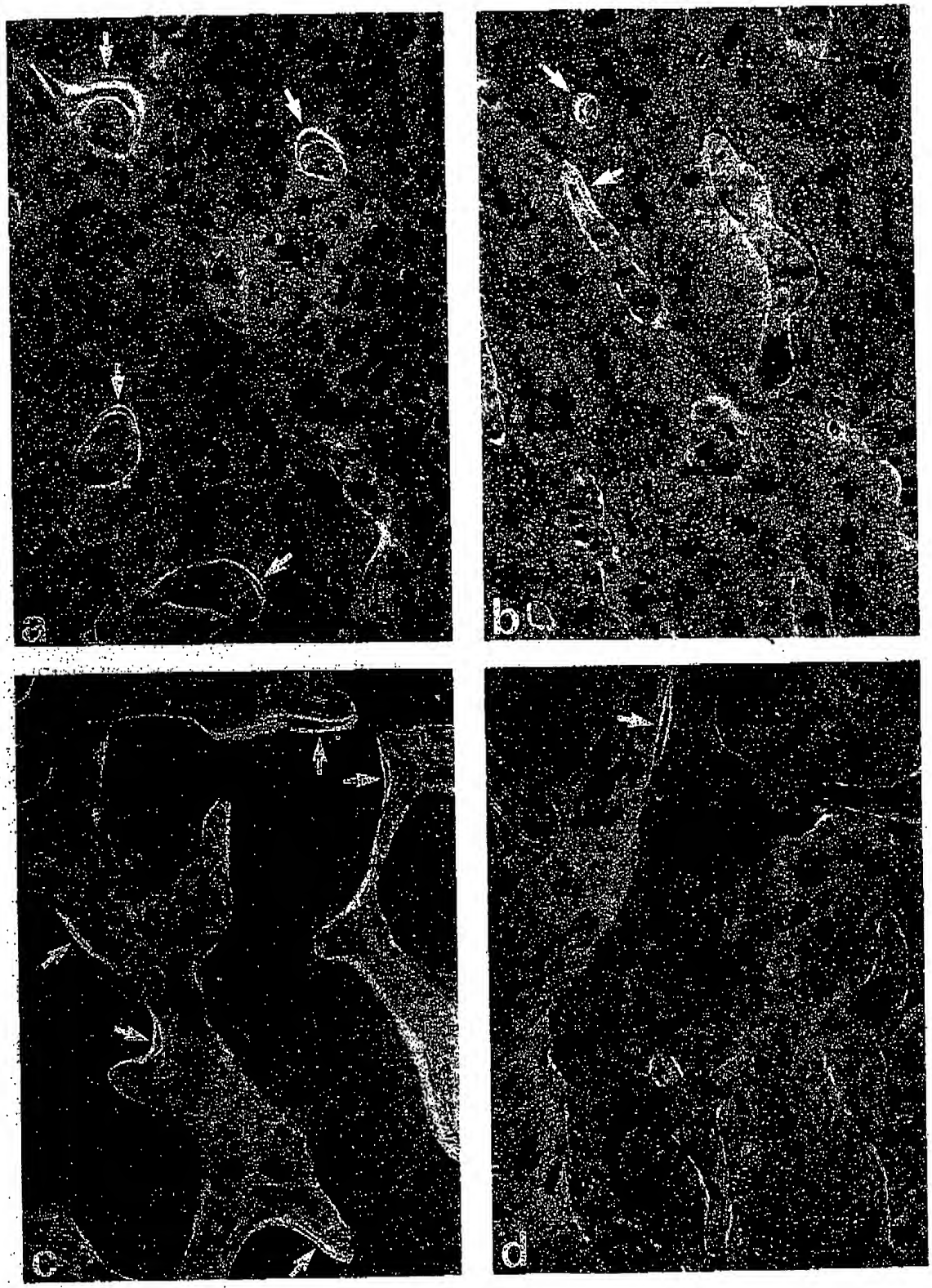

Fig. 1. Trabecular bone tissue located 2–3 mm from the growth plate–metaphyseal junction in the proximal tibiae of ovariectomized (**a**) and control (**b**) rats. Note the increased incidence of double tetracycline labels (arrows) indicative of increased active formation surface in the ovariectomized animal. The difference in double tetracycline labeling in equivalent regions of the lumbar vertebrae of ovariectomized (**c**) and control (**d**) rats is also apparent. UV illumination. ×50.

Table III. Histomorphometric parameters at the cortical endosteal surface of the proximal tibia.

	Osteoid seam width (μm)	Calcification rate (μm/day)	Mineralization lag time (days)
Ovariectomized* ($n=13$)	6.7 ±1.6	5.0 ±1.0	1.4 ±0.3
Control ($n=9$)	5.6 ±1.9	3.9 ±1.4	1.4 ±0.4

*No significant differences were observed between ovariectomized and control rats.

Oophorectomized women also exhibit biochemical evidence for increased bone turnover as early as 6 weeks after surgery (Fogelman et al., 1984). With the reservation that rats have a juvenile skeleton in which bone resorption and formation are not necessarily coupled, our histomorphometric study indicates that accelerated bone turnover also occurs in rats at early times postovariectomy.

In summary, ovariectomized rats are characterized by osteopenia and histologic evidence for increased bone turnover. The osteopenic changes are more pronounced in the proximal tibia relative to the lumbar vertebra. These findings are consistent with reports of accelerated skeletal metabolism in postmenopausal and oophorectomized women during the early stages of estrogen deficiency.

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OVX → both BF & Resorp ↑

estrogen / bisphos → ↓ both BF & BR

Estrogen and Diphosphonate Treatment Provide Long-Term Protection Against Osteopenia in Ovariectomized Rats

T.J. WRONSKI, C.-F. YEN, and K.S. SCOTT

ABSTRACT

The goal of this study is to determine whether the previously observed, short-term protective effect of estrogen and diphosphonate compounds against osteopenia in ovariectomized (OVX) rats can be maintained for an entire year. Sham-operated control and OVX rats were treated intermittently with vehicle alone, estrogen, or the diphosphonate compounds etidronate disodium (EHDP) and risedronate (NE-58095) for 360 days after surgery. Their proximal tibiae and first lumbar vertebrae were processed undecalcified for quantitative bone histomorphometry. Both skeletal sites in vehicle-treated OVX rats were characterized by decreased cancellous bone volume and increases in most cellular and fluorochrome-based indices of bone formation and resorption. Treatment of OVX rats with estrogen or diphosphonate compounds depressed bone turnover and provided nearly complete protection against cancellous bone loss. Long-term EHDP treatment induced a moderate mineralization defect, as indicated by increased absolute osteoid volume and a high proportion of osteoid surfaces devoid of adjacent osteoblasts. In contrast, NE-58095 had minimal effects on bone mineralization. These findings indicate that diphosphonate compounds and estrogen provide long-term protection against tibial and vertebral osteopenia in OVX rats. They further indicate that diphosphonate compounds merit consideration as an alternative to estrogen for the prevention of postmenopausal bone loss.

INTRODUCTION

Postmenopausal osteoporosis and the resulting increased incidence of bone fractures is a serious health problem in elderly women. At the present time estrogen replacement is the only widely accepted preventive measure for this bone disorder. Estrogenic suppression of bone turnover appears to be the major mechanism for this protective effect.[1–4] It follows that other endocrine or pharmacologic suppressors of bone turnover may also be effective in the prevention of postmenopausal bone loss. This hypothesis was recently tested with the ovariectomized rat as an animal model for estrogen deficiency. Diphosphonate compounds were found to be as effective as estrogen in depressing bone turnover and protecting against osteopenia during the first several months postovariectomy.[5] However, the question arises whether the skeletal integrity of ovariectomized rats can be maintained by diphosphonates over a longer treatment period without adverse side effects, such as impaired bone mineralization. Therefore, the current report consists of a histomorphometric characterization of the skeleton of ovariectomized rats treated for an entire year with estrogen or diphosphonate compounds. The potential bone-protective effect of such treatments was studied at both axial and appendicular skeletal sites in ovariectomized rats.

MATERIALS AND METHODS

Female Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). These animals were 90 days of age and weighed an average of 240 g at the beginning of the study. All rats were anesthetized with an intraperitoneal (IP) injection of ketamine hydro-

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chloride and xylazine at doses of 50 and 10 mg/kg body weight, respectively. Bilateral ovariectomies were performed in half the rats from a dorsal approach. The remainder were subjected to sham surgeries in which the ovaries were exteriorized but not removed. Ovariectomized (OVX) and sham-operated control rats were divided into various groups based on treatment with estrogen, diphosphonate compounds, or vehicle alone. For estrogen treatments, 17β-estradiol (Sigma Chemical Co., St. Louis, MO) was dissolved in a vehicle of 95% corn oil and 5% benzyl alcohol. The diphosphonate compounds were obtained from Norwich Eaton Pharmaceuticals, Inc. (Norwich, NY). Etidronate disodium (EHDP, ethane-1-hydroxy-1,1-diphosphonate), an established diphosphonate, and risedronate [NE-58095, 2-(3-pyridinyl)-2-hydroxyethylidene-1,1-bisphosphonate disodium], a new, highly potent, nitrogen-containing diphosphonate, were dissolved in a saline vehicle. Each of the following groups consisted of 10–12 OVX rats and 10–12 sham-operated control rats.

1. Vehicle. Half of the OVX and control rats in this group were injected subcutaneously (SC) 5 days/week with the corn oil and benzyl alcohol vehicle used for estrogen treatment. The remainder were injected SC with the saline vehicle used for diphosphonate treatment. Within the saline-treated group half the OVX and control rats were injected according to the 1 week on, 3 weeks off regimen (see regimen 1); the remainder were injected with saline twice weekly (regimen 2).
2. Estrogen. Each OVX and control rat in this group was injected SC with 17β-estradiol, 5 days/week, at a dose of 10 μg/kg body weight.
3. EHDP (regimen 1). Each OVX and control rat in this group was injected SC with EHDP at a dose of 5 mg/kg body weight (1.25 mg P). Treatments were performed daily for a 1 week period, followed by a 3 week period without treatment. This cycle was repeated for the duration of the study. This regimen is referred to as the 1 week on, 3 weeks off regimen.
4. EHDP (regimen 2). Each OVX and control rat in this group was injected SC with EHDP twice weekly at a dose of 5 mg/kg body weight (1.25 mg P).
5. NE-58095. Each OVX and control rat in this group was injected SC with NE-58095 at a dose of 5 μg/kg body weight (1 μg P) according to the 1 week on, 3 weeks off regimen.

All of these treatments were initiated on the first day after surgery. The rats were housed individually at 25°C with a 13 h/11 h light-dark cycle. Food (Purina Rat Laboratory Chow, St. Louis, MO) was available ad libitum to the sham-operated control rats. Their food consumption was measured and a daily mean calculated for each control group (~20 g/day per rat). The daily food consumption of OVX rats was restricted to the mean for the corresponding control rats (pair feeding) to minimize the increase in body weight associated with ovariectomy.[6] Each rat was injected IP with demeclocycline (Lederle Laboratories, Pearl River, NY) and calcein (Sigma Chemical Co., St. Louis, MO) on days 17 and 7 before sacrifice, respectively. This regimen resulted in deposition of a double-fluorochrome label at bone surfaces that were actively mineralizing throughout the injection period.

A group of 10 baseline control rats was sacrificed by exsanguination under ketamine-xylazine anesthesia on the day of surgery (day 0). All other rats were sacrificed 360 days after surgery. The success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns. Both proximal tibiae and the first lumbar vertebrae from each animal were stripped of musculature and placed in 10% phosphate-buffered formalin for 24 h. The bone specimens were then dehydrated in ethanol and embedded undecalcified in methyl methacrylate.[7] Longitudinal sections of 4 μm thickness were cut with an AO Autocut/Jung 1150 microtome and stained according to the Von Kossa method with a tetrachrome counterstain (Polysciences Inc., Warrington, PA). Bone measurements were performed in cancellous bone tissue of the proximal tibial metaphysis at distances greater than 1 mm from the growth plate-metaphyseal junction to exclude the primary spongiosa. Similarly, the sample site in the lumbar vertebral body excluded cancellous bone tissue within 1 mm of the cranial and caudal growth plates. Additional details of the sample sites have been published elsewhere.[8,9] In general, two sections each of the proximal tibia and lumbar vertebra were sampled in each control animal. These sample sites consisted of 40–60 mm of cancellous bone perimeter within each bone. The relative lack of cancellous bone spicules in the proximal tibia of OVX rats made it necessary to sample additional sections at this skeletal site. Therefore, surface-based measurements were performed in three or more tibial sections in each OVX rat to approximate the cancellous bone perimeter sampled in control rats.

Bone measurements were performed with the Bioquant Bone Morphometry System (R & M Biometrics Corp., Nashville, TN). Cancellous bone areas and surface lengths were traced with a cursor on a Hipad digitizing tablet adjacent to a Nikon Labophot microscope. The light within the cursor can be visualized in the microscopic field when used in conjunction with a camera lucida. Raw data were stored in an Apple IIe microcomputer interfaced to the digitizing tablet. Bone histomorphometric values were then calculated with Bioquant software. Cancellous bone volume and absolute osteoid volume were measured as percentages of the total cancellous bone tissue area. Osteoblast, osteoclast, and osteoid surface were measured as percentages of total cancellous bone surface length. In addition, osteoid surface without adjacent osteoblasts (inactive osteoid) was measured as a percentage of total osteoid surface.

Fluorochrome-based indices of bone formation were measured in unstained, 8 μm thick sections of the proximal tibial metaphysis and lumbar vertebral body. The percentage of cancellous bone surface with a double-fluorochrome label (mineralizing surface) and mineral apposition rate were measured with the Bioquant system. In addition, bone formation rate (tissue level, total surface referent) was calculated by multiplying the mineralizing surface by the mineral apposition rate.[10] Values for mineral apposi-

tion rate were not corrected for obliquity of the plane of section in cancellous bone.[10]

Measurement of cancellous bone volume and specific surface in the baseline control animals allowed the calculation of bone balance.[10] Bone resorption rate (tissue level, total surface referent) was determined by subtracting bone balance from bone formation rate.[10]

Data are expressed as the mean ± SD (standard deviation) for each group. Statistical differences among groups were evaluated with the Kruskal-Wallis test.[11] P values < 0.05 were considered significant.

RESULTS

Within the groups of vehicle-treated OVX and control rats, the type of vehicle (corn oil or saline) and the different saline regimens did not induce statistically significant changes in body weight or bone histomorphometric values. Therefore the data were combined within the vehicle-treated OVX group as well as within the vehicle-treated control group.

All rats gained substantial weight during the yearlong duration of the study. The mean body weights for the 10 groups of rats ranged from 390 to 455 g, with no statistically significant differences among the groups.

Table 1 lists bone histomorphometric data from cancellous bone tissue in the proximal tibial metaphysis of the 10 groups of rats. Vehicle-treated OVX rats exhibited a marked decrease in cancellous bone volume and statistically significant increases in osteoblast surface, osteoid surface, bone formation rate, and bone resorption rate relative to vehicle-treated control rats. A trend for increased osteoclast surface was observed in vehicle-treated OVX rats, but this trend was not statistically significant. In contrast, cancellous bone volume in estrogen-treated OVX rats was at least a factor of 3 greater than that of vehicle-treated OVX rats. Estrogen treatment of OVX rats also markedly decreased osteoblast surface, osteoid surface, bone formation rate, and bone resorption rate to levels comparable to those of vehicle-treated control rats. Treatment of sham-operated control rats with estrogen did not significantly alter cancellous bone volume or histomorphometric indices of bone turnover relative to vehicle-treated control rats.

OVX rats treated with EHDP according to the 1 week on, 3 weeks off regimen were found to have a cancellous bone volume approximately threefold greater than that of vehicle-treated OVX rats. In addition, significant decreases in osteoblast surface, osteoclast surface, osteoid surface, bone formation rate, and bone resorption rate were detected in these animals. Treatment of OVX rats twice weekly with EHDP resulted in a greater cancellous bone volume relative to vehicle-treated OVX rats and statistically significant decreases in all the bone parameters, with the exception of osteoid surface. OVX and control rats treated with either EHDP regimen exhibited marked increases in inactive osteoid. Absolute osteoid volume was 2.3 ± 3.2% and 0.5 ± 0.6% in OVX and control rats, respectively, treated with EHDP twice weekly. These values were significantly greater ($P < 0.05$) than the mean absolute osteoid volumes in all other groups (≤0.1%).

Treatment of OVX rats with NE-58095 according to the 1 week on, 3 weeks off regimen resulted in the following bone changes relative to vehicle-treated OVX rats: greater cancellous bone volume, decreased osteoblast and osteoid surface, and decreased bone formation and resorption rates. These animals also exhibited a moderate but statistically nonsignificant trend for decreased osteoclast surface. In contrast to EHDP-treated OVX rats, treatment with NE-58095 did not induce increases in tibial absolute osteoid volume or inactive osteoid.

Sham-operated control rats treated with either EHDP or NE-58095 exhibited marked increases in tibial cancellous bone volume and decreased indices of bone formation and resorption. Their values for cancellous bone volume were approximately a factor of 3 greater than diphosphonate-treated OVX rats and a factor of 2 greater than vehicle-treated control rats.

Bone histomorphometric data from cancellous bone tissue in the first lumbar vertebral body are listed in Table 2. In general, bone changes in the lumbar vertebrae of vehicle-treated OVX rats were similar to those observed in the proximal tibiae. These changes include decreased cancellous bone volume and increases in the following parameters: osteoblast surface, osteoclast surface, osteoid surface, bone formation rate, and bone resorption rate. Treatment of OVX rats with estrogen normalized cancellous bone volume and decreased all the indices of bone turnover to levels comparable to those of vehicle-treated control rats. Normal cancellous bone volume and decreased bone turnover were also observed in OVX rats treated with EHDP either according to the 1 week on, 3 weeks off regimen or twice weekly. However, these latter animals did not exhibit a decline in osteoid surface relative to vehicle-treated OVX rats and their mean value for inactive osteoid was significantly greater than the means for the vehicle-treated control and OVX rats. A similar increase in inactive osteoid was detected in OVX rats treated with EHDP according to the 1 week on, 3 weeks off regimen. Cancellous bone volume in OVX rats treated with NE-58095 was significantly greater than that of vehicle-treated OVX rats but significantly less than that of vehicle-treated control rats. NE-58095 decreased osteoblast and osteoid surfaces as well as bone formation and resorption rates in OVX rats. However, osteoclast surface and inactive osteoid were not significantly different in OVX rats treated with vehicle or NE-58095.

Differences in vertebral cancellous bone volume among rats of the various treatment groups are seen in Fig. 1.

Absolute osteoid volume was <0.1% in the lumbar vertebrae of all groups of rats except those treated with EHDP. OVX and control rats treated with EHDP according to the 1 week on, 3 weeks off regimen had mean absolute osteoid volumes of 0.2 ± 0.2% and 0.3 ± 0.2%, respectively; OVX and control rats treated with EHDP twice weekly had means of 2.7 ± 2.5% and 4.7 ± 2.9%, respectively. The excess osteoid induced by EHDP treatment can

TABLE 1. BONE HISTOMORPHOMETRIC DATA IN THE PROXIMAL TIBIAL METAPHYSIS AT 360 DAYS POSTSURGERY[a]

	Control + VEH	*OVX + VEH*	*Control + EST*	*OVX + EST*	*Control + EH1*	*OVX + EH1*	*Control + EH2*	*OVX + EH2*	*Control + NE-5*	*OVX + NE-5*
Bone volume, %	21.3[b] ± 11.0	4.7[c] ± 4.1	25.1[b] ± 9.1	16.5[b] ± 5.6	38.2[b,c] ± 10.6	14.8[b,c] ± 8.1	42.8[b,c] ± 8.8	13.3[b,c] ± 6.0	52.3[b,c] ± 9.9	16.8[b] ± 9.6
Osteoblast surface, %	2.4[b] ± 1.4	10.5[c] ± 3.9	2.3[b] ± 2.2	2.4[b] ± 1.3	0.1[b,c] ± 0.1	0.3[b,c] ± 0.5	0.2[b,c] ± 0.2	2.2[b] ± 3.3	0.3[b,c] ± 0.3	1.7[b] ± 1.7
Osteoclast surface, %	1.7 ± 1.0	2.6 ± 1.2	1.4[b] ± 0.8	2.1 ± 0.8	0.4[b,c] ± 0.3	1.1[b] ± 0.6	0.5[b,c] ± 0.3	1.2[b] ± 0.7	1.3[b] ± 1.0	1.8 ± 0.6
Osteoid surface, %	1.5[b] ± 1.0	8.6[c] ± 3.3	1.6[b] ± 1.5	1.7[b] ± 0.9	0.2[b,c] ± 0.2	2.1[b] ± 3.5	2.0[b] ± 1.9	11.6 ± 13.8	0.2[b,c] ± 0.3	1.4[b] ± 1.5
Inactive osteoid, %[d]	0.0 ± 0.0	0.0 ± 0.0	3.3 ± 11.5	1.0 ± 3.6	30.6[b,c] ± 38.2	72.1[b,c] ± 29.4	69.4[b,c] ± 35.5	68.7[b,c] ± 26.1	0.0 ± 0.0	1.1 ± 3.3
Bone formation rate, × 10^{-3} $\mu m^3/\mu m^2$/day	0.3[b] ± 0.4	2.1[c] ± 2.2	0.1[b] ± 0.1	0.1[b] ± 0.1	<0.1[b]	<0.1[b]	<0.1[b]	<0.1[b]	<0.1[b]	<0.1[b]
Bone resorption rate, × 10^{-3} $\mu m^3/\mu m^2$/day	0.3[b] ± 0.5	2.3[c] ± 2.2	0.1[b] ± 0.1	0.1[b] ± 0.1	0.0[b,c] ± 0.0	0.2[b] ± 0.1	0.0[b,c] ± 0.0	0.2[b] ± 0.1	0.0[b,c] ± 0.0	0.2[b] ± 0.1

[a]All values are the mean ± SD. Control and OVX rats were treated with vehicle alone (VEH), 17β-estradiol (EST), EHDP according to the 1 week on, 3 weeks off regimen (EH1), EHDP twice weekly (EH2), or NE-58095 (NE-5).

[b]Significantly different from vehicle-treated OVX value ($P < 0.05$).

[c]Significantly different from vehicle-treated control value ($P < 0.05$).

[d]Defined as osteoid surface without adjacent osteoblasts.

TABLE 2. BONE HISTOMORPHOMETRIC DATA IN THE FIRST LUMBAR VERTEBRAL BODY AT 360 DAYS POSTSURGERY[a]

	Control + VEH	*OVX + VEH*	*Control + EST*	*OVX + EST*	*Control + EH1*	*OVX + EH1*	*Control + EH2*	*OVX + EH2*	*Control + NE-5*	*OVX + NE-5*
Bone volume, %	40.5[b] ± 5.5	25.2[c] ± 3.4	49.7[b,c] ± 6.5	45.4[b] ± 6.5	49.5[b,c] ± 6.5	39.2[b] ± 9.8	45.3[b] ± 9.4	36.0[b] ± 5.9	42.0[b] ± 7.3	34.1[b,c] ± 5.5
Osteoblast surface, %	2.9[b] ± 1.3	10.4[c] ± 4.5	2.4[b] ± 1.9	1.9[b] ± 0.7	1.3[b,c] ± 1.4	0.8[b,c] ± 1.2	3.8[b] ± 2.2	3.1[b] ± 2.8	0.6[b,c] ± 0.8	0.7[b,c] ± 0.9
Osteoclast surface, %	0.3[b] ± 0.2	1.1[c] ± 0.6	0.2[b] ± 0.2	0.3[b] ± 0.2	0.2[b] ± 0.2	0.5[b] ± 0.4	0.2[b] ± 0.1	0.2[b] ± 0.2	0.3[b] ± 0.3	0.7[c] ± 0.5
Osteoid surface, %	1.9[b] ± 0.9	8.7[c] ± 3.6	1.9[b] ± 1.4	1.4[b] ± 0.5	6.3[c] ± 3.2	2.9[b] ± 3.1	26.9[b,c] ± 11.3	15.5[c] ± 12.6	1.2[b] ± 1.2	0.7[b,c] ± 1.1
Inactive osteoid, %[d]	0.0 ± 0.0	0.1 ± 0.4	4.5 ± 14.4	1.4 ± 4.8	79.8[b,c] ± 15.3	55.3[b,c] ± 31.4	85.2[b,c] ± 8.0	78.3[b,c] ± 13.6	39.5[b,c] ± 30.1	9.8 ± 19.9
Bone formation rate, × 10^{-2} $\mu m^3/\mu m^3$/day	1.4 ± 1.3	2.1 ± 1.1	0.7[b] ± 0.5	0.6[b] ± 0.3	<0.1[b,c]	<0.1[b,c]	<0.1[b,c]	<0.1[b,c]	<0.1[b,c]	<0.1[b,c]
Bone resorption rate, × 10^{-2} $\mu m^3/\mu m^3$/day	1.3 ± 1.3	2.2 ± 1.2	0.6[b] ± 0.5	0.6[b] ± 0.3	<0.1[b,c]	<0.1[b,c]	<0.1[b,c]	<0.1[b,c]	<0.1[b,c]	<0.1[b,c]

[a]All values are the mean ± SD. Control and OVX rats were treated with vehicle alone (VEH), 17β-estradiol (EST), EHDP according to the 1 week on, 3 weeks off regimen (EH1), EHDP twice weekly (EH2), or NE-58095 (NE-5).

[b]Significantly different from vehicle-treated OVX value ($P < 0.05$).

[c]Significantly different from vehicle-treated control value ($P < 0.05$).

[d]Defined as osteoid surface without adjacent osteoblasts.

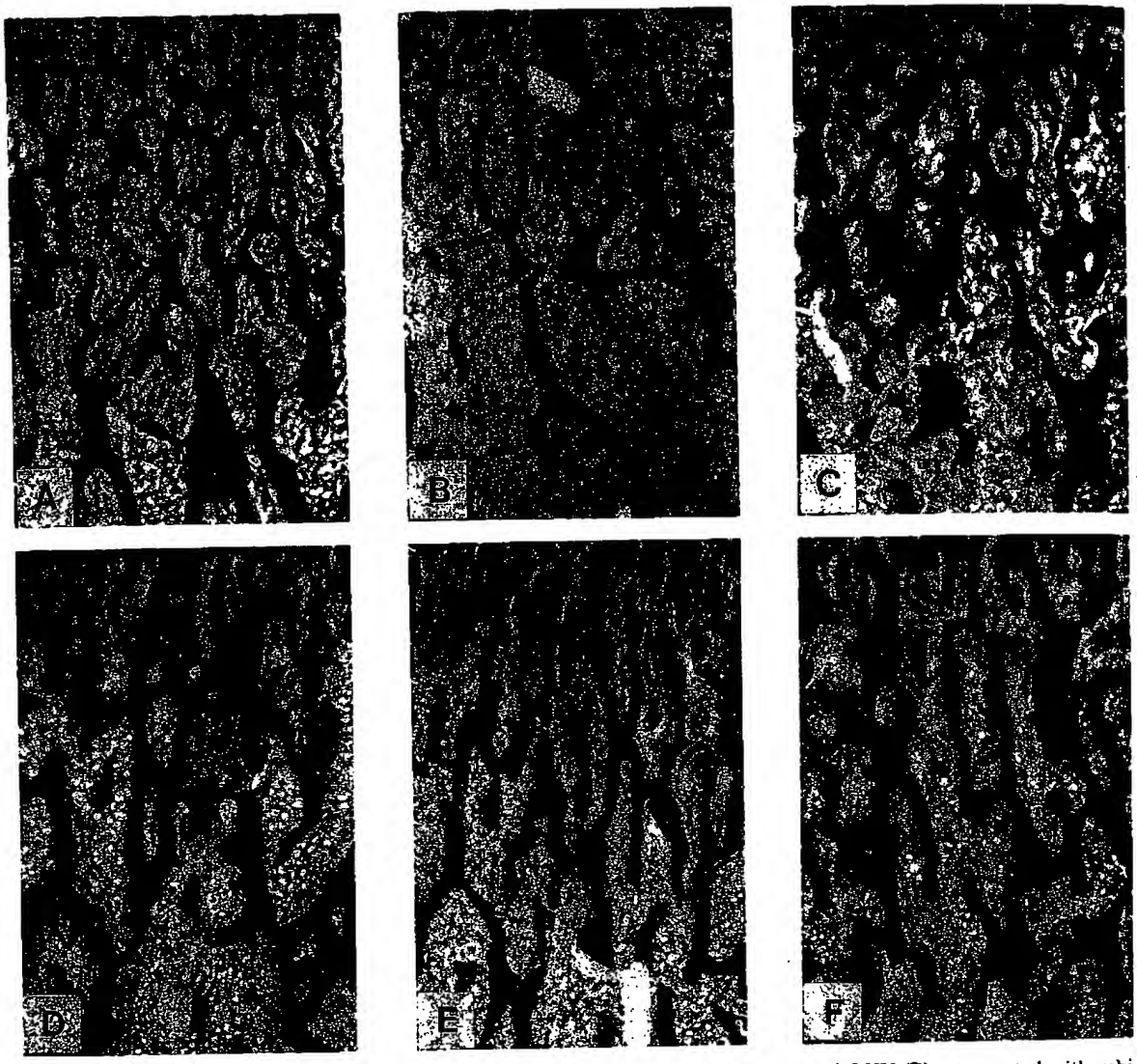

FIG. 1. Cancellous bone tissue in the first lumbar vertebral body from control (A) and OVX (B) rats treated with vehicle alone, OVX rats treated with estrogen 5 days/week (C) or EHDP twice weekly (D), and OVX rats treated with EHDP (E) or NE-58095 (F) according to the 1 week on, 3 weeks off regimen. The top of each photomicrograph is situated at 1 mm from the cranial growth plate so that equivalent areas of cancellous bone tissue are shown in each animal. Note the reduced amount of darkly stained cancellous bone in the vehicle-treated OVX rat. Note also the increased amount of cancellous bone in OVX rats treated with estrogen or diphosphonate compounds. Von Kossa stain, original magnification ×15.

be seen in Fig. 2. In contrast, OVX and control rats treated with the other diphosphonate compound, NE-58095, had mean absolute osteoid volumes <0.1%.

The lumbar vertebrae of sham-operated control rats treated with estrogen or diphosphonate compounds exhibited trends for increased cancellous bone volume relative to the vertebrae of vehicle-treated control rats. However, these trends were not statistically significant, with the exception of control rats treated with EHDP according to the 1 week on, 3 weeks off regimen. Control rats treated with either EHDP or NE-58095 had significant increases in inactive osteoid.

DISCUSSION

We previously reported that treatment of OVX rats with estrogen or diphosphonate compounds suppresses bone turnover and protects against tibial osteopenia for the first several months postovariectomy.[5] This finding provided preliminary evidence for consideration of diphosphonate compounds as an alternative to estrogen for the prevention of postmenopausal bone loss. However, the question arises whether the protective effect of diphosphonate compounds can be maintained over a longer treatment period. The current study demonstrates that diphosphonate compounds

FIG. 2. Cancellous bone tissue in the first lumbar vertebral body of an OVX rat treated with EHDP twice weekly. Note the areas of excess osteoid (arrows) indicative of impaired bone mineralization. Von Kossa stain, ×150.

and estrogen are equally effective in preserving cancellous bone mass for an entire year in OVX rats. This protective effect was associated with decreased bone turnover and observed in both the appendicular and axial skeletons of OVX rats. This latter finding is especially significant because of the presence of at least some bone remodeling in the rat vertebral column, which makes it more comparable to adult human bone.[12]

The diphosphonate compound etidronate (EHDP) has recently been shown to depress bone turnover, increase spinal bone mineral density, and reduce vertebral fracture rates in patients with established osteoporosis.[13–15] However, clinical trials of the use of diphosphonates specifically for the prevention of early postmenopausal bone loss are sparse. Smith et al.[16] reported that EHDP depresses bone turnover soon after surgical menopause, but the duration of their study was too short to evaluate the effect of such treatment on bone mass. However, a recent clinical trial indicated that diphosphonates effectively prevent bone loss in early postmenopausal women.[17] These findings in estrogen-deficient women are consistent with the skeletal effects of diphosphonates in OVX rats and further support the concept of diphosphonates as an alternative to estrogen for the prevention of postmenopausal bone loss.

High doses of certain diphosphonate compounds are known to impair bone mineralization.[18–20] In our previous short-term study of 1–2 months' duration,[5] low doses of diphosphonates given intermittently induced only a slight mineralization defect. It is therefore of particular interest to determine whether the extended treatment period of the current study (~1 year) impaired bone mineralization in OVX and intact, control rats. The observed increases in absolute osteoid volume and inactive osteoid indicate that EHDP induced a mineralization defect. The increase in the latter parameter provides evidence that most osteoid surfaces in EHDP-treated rats are devoid of osteoblasts, which is a rare occurrence in normal rats as a result of their short mineralization lag time of 1–4 days.[5,6] Since inactive osteoid was rarely detected in vehicle-treated rats, we interpret the high incidence of inactive osteoid in EHDP-treated rats as a consequence of a prolonged mineralization lag time. The mineralization defect was slight in OVX and control rats treated with EHDP according to the 1 week on, 3 weeks off regimen but was more pronounced in rats treated with EHDP twice weekly. Furthermore, the mineralization defect induced by EHDP tended to be more pronounced in the lumbar vertebra relative to the proximal tibia. On the other hand, the effect of the new diphosphonate compound, NE-58095, on bone mineralization was minimal as evidenced by normal values for tibial absolute osteoid volume and inactive osteoid in most rats treated with the drug. The exception was the significant increase in vertebral inactive osteoid in sham-operated control rats treated with NE-58095. This finding suggests that long-term treatment with NE-58095 may induce a slight alteration in bone mineralization. Nevertheless, it is important to note that NE-58095 provided nearly complete protection against osteopenia in OVX rats with minimal impairment of bone mineralization at a dose a thousandfold less than that of EHDP.

Caution should be exercised in extrapolating between rats and humans on the effects of diphosphonates on bone mineralization. Although the dose of EHDP (5 mg/kg body weight) administered to rats in the current study and to humans in clinical trials is nearly identical, the route of administration differs (SC versus oral, respectively). Since diphosphonates are poorly absorbed through the gastrointestinal tract,[21] oral administration of the drug presumably results in decreased skeletal uptake and less likelihood for impaired bone mineralization. This appears to be the case, as humans treated orally with EHDP (5–10 mg/kg body weight) according to an intermittent, cyclical regimen (2 weeks on, 10–13 weeks off) for periods as long as 150 weeks in recent clinical trials did not exhibit any signs of osteomalacia.[14,15]

In summary, the current study demonstrates that treat-

ment of OVX rats with estrogen or diphosphonate compounds provides long-term protection against the development of both tibial and vertebral osteopenia. This protective effect is associated with decreased bone turnover at both skeletal sites. Despite the relatively long treatment period (~1 year), the effects of the new diphosphonate compound, NE-58095, on bone mineralization were minimal, whereas EHDP induced a moderate mineralization defect. These findings support the concept of diphosphonate compounds as an alternative to estrogen for the prevention of postmenopausal bone loss.

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Skeletal Effects of Calcitonin Treatment and Withdrawal in Ovariectomized Rats

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Abstract. The study was designed to determine, by histomorphometric techniques, bone changes as a function of time during long-term treatment with salmon calcitonin (CT) and after withdrawal of the hormone in ovariectomized (OVX) rats. Groups of OVX rats were treated with vehicle alone or CT on alternate days for 30, 60, or 90 days. Additional groups of sham-operated control rats were treated with vehicle alone. Rats from each of the three groups were sacrificed at each time point. All treatments in the remaining rats were then terminated at 90 days, followed by sacrifice of rats from each group at 30 and 60 days after withdrawal of vehicle or CT treatment. The proximal tibia from each animal was processed undecalcified for quantitative bone histomorphometry. Compared with control rats, the proximal tibiae of vehicle-treated OVX rats were characterized by cancellous osteopenia and significant increases in osteoclast surface, osteoblast surface, mineralizing surface, mineral apposition rate, and bone formation rate. CT treatment of OVX rats partially prevented cancellous bone loss by approximately 50% and significantly decreased most of the above indices of bone turnover relative to vehicle-treated OVX rats. However, soon after withdrawal of CT, OVX rats previously treated with the hormone exhibited rapid loss of cancellous bone associated with increased bone turnover. These results in an animal model of estrogen depletion suggest that early postmenopausal women who are withdrawn from prophylactic CT treatment may be at high risk for subsequent bone loss.

Key words: Ovariectomy — Cancellous bone — Histomorphometry — Osteopenia — Calcitonin withdrawal.

Postmenopausal osteoporosis is characterized by a reduction in bone mass associated with an increase in bone turnover and an imbalance between bone resorption and formation [1]. Calcitonin (CT), a peptide hormone, decreases bone resorption acutely [2, 3]. Clinical studies have demonstrated its efficacy for the prevention of postmenopausal bone loss [4–9]. A modest increase in bone mass has also been observed after CT treatment in patients with established osteoporosis [10–13]. The consensus of these studies is that the beneficial effects of the hormone on bone mass are due to a suppression of bone turnover.

Continuous treatment of postmenopausal women for several decades with CT is not practical due to the prohibitive cost of the hormone. Therefore, the skeletal response to withdrawal of CT treatment is of considerable interest. In the only such clinical study to date, Overgaard et al. [14] reported that, after withdrawal of CT, the rate of bone loss in osteoporotic patients previously treated with the hormone was similar to that of placebo-treated patients. Biochemical estimates of bone turnover were also found to increase soon after CT withdrawal.

A more complete characterization by bone histomorphometric techniques of the skeletal effects of CT treatment and withdrawal is highly desirable. Since such invasive studies are rare in humans, animal models are often relied upon to provide bone histomorphometric data. In this regard, the ovariectomized (OVX) rat has been widely used as an animal model for early postmenopausal bone loss. The skeletal response to estrogen depletion is similar in OVX rats and early postmenopausal women [15, 16]. Furthermore, much as in postmenopausal women, CT has been shown to depress bone turnover and provide at least partial protection against bone loss in OVX rats [17, 18]. However, the skeletal response to withdrawal of CT treatment in OVX rats has not been reported to date.

Therefore, the objectives of the present study were to determine by histomorphometric techniques changes in cancellous bone mass and turnover as a function of time during long-term treatment with CT as well as bone changes after withdrawal of CT in estrogen-deplete rats.

Materials and Methods

The experimental animals were 144 female Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA) that were approximately 90 days of age and weighed an average of 220 g at the beginning of the study. Eight rats were sacrificed on the day of surgery (day 0) as baseline controls. The remaining rats were anesthetized with an I.P. injection of ketamine hydrochloride and xylazine at doses of 50 and 10 mg/kg body weight, respectively. Forty-six rats were subjected to sham surgeries in which the ovaries were exteriorized but replaced intact. Bilateral ovariectomies were performed from a dorsal approach in the remaining 90 rats. Each rat was housed individually at 25°C with a light/dark cycle of 13 hours/11 hours. Food (Teklad 22/5 Rodent Diet, Madison, WI), with Ca and PO_4 contents of 0.95% and 0.67%, respectively, was available *ad libitum* to the sham-operated control rats. The food consumption of OVX rats was restricted to that of control rats (pair-feeding) to minimize the increase in body weight associated with ovariectomy [19].

One group of OVX rats was injected S.C. with salmon calcitonin (Bachem Inc., Torrance, CA) on alternate days at a dose of 4 μg/kg body weight (16 U/kg body weight). Sham-operated con-

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trol rats and the remaining OVX rats were injected S.C. on alternate days with saline vehicle. These treatments were initiated on the first day after surgery and continued for periods of 30, 60, or 90 days. Vehicle- or CT-treated rats from each of the three groups (n = 8–10 per group) were sacrificed at each time point. After 90 days of treatment with vehicle or CT, treatments were withdrawn in the remaining rats followed by sacrifice of six rats from each group at 30 and 60 days after withdrawal. Prior to sacrifice, all rats were injected I.P. with demeclocycline (Lederle Laboratories, Pearl River, NY) and calcein (Sigma Chemical Co., St. Louis, MO) at a dose of 15 mg/kg body weight on the 10th and 3rd days prior to sacrifice, respectively, to label sites of bone formation.

All rats were sacrificed by exsanguination from the abdominal aorta under ketamine/xylazine anesthesia. Success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns. The right proximal tibia from each animal was stripped of musculature and placed in 10% phosphate-buffered formalin for 24 hours for tissue fixation. Soon after their collection, serum samples were stored at −80°C until analyzed spectrophotometrically for their calcium content with a CIBA-Corning 550 Express Random Access Chemistry Analyzer (Oberlin, OH) by the cresolphthalein complexone colorimetric method.

Bone samples were dehydrated in ethanol and embedded undecalcified in methyl methacrylate [20]. Longitudinal sections (4- and 8-μm thick) were cut with an AO Autocut/Jung 1150 microtome. The 4-μm thick sections were stained according to the Von Kossa method with a tetrachrome counterstain (Polysciences Inc., Warrington, PA). Bone measurements were performed in cancellous bone tissue of the proximal tibial metaphysis at distances greater than 1 mm from the growth plate-metaphyseal junction to exclude the primary spongiosa. In general, two sections of the proximal tibia with 40–50 mm of cancellous bone perimeter were sampled in each animal with an appreciable amount of cancellous bone. Additional sections were sampled in osteopenic animals to approximate the cancellous bone perimeter sampled in animals with greater cancellous bone mass.

Bone measurements were performed with the Bioquant Bone Morphometry System (R&M Biometrics Corp., Nashville, TN) as previously described [21]. Cancellous bone volume as a percentage of bone tissue area and osteoblast and osteoclast surfaces as percentages of total cancellous perimeter were measured in 4-μm thick, stained sections.

Fluorochrome-based indices of bone formation were measured in unstained, 8-μm-thick sections of the proximal tibial metaphysis. The percentage of cancellous bone surface with a double fluorochrome label (mineralizing surface) and mineral apposition rate were measured with the Bioquant system. In addition, bone formation rate (tissue level, total surface referent) was calculated by multiplying mineralizing surface by mineral apposition rate [22]. Values for mineral apposition rate were not corrected for obliquity of the plane of section in cancellous bone [23].

Data are expressed as the mean ± SE for each group. Statistical differences among groups were evaluated at each time point with the Kruskal Wallis test involving a multiple comparison procedure [24]. P values less than 0.05 were considered to be significant.

Results

All groups of rats gained weight during the course of the study as their mean values increased from approximately 220 g on day 0 to 300–320 g at the end of the 90-day treatment period. Despite pair-feeding, vehicle-treated OVX rats tended to gain more body weight than vehicle-treated control rats. On the other hand, CT-treated OVX rats weighed significantly less ($P < 0.05$) than vehicle-treated OVX rats at 60 and 90 days postovariectomy. After withdrawal of CT treatment, the body weights of OVX rats previously treated with the hormone were not restored to the level of vehicle-treated OVX rats.

No significant differences in serum calcium were observed among the three treatment groups. For example, on day 30, mean serum calcium values for vehicle-treated control, vehicle-treated OVX, and CT-treated OVX rats were 10.9 ± 0.3, 10.8 ± 0.3, and 10.3 ± 0.2 mg/dl, respectively. Although the relatively normal value for CT-treated OVX rats suggests that these animals were not hypocalcemic, it is important to note that the terminal blood samples were collected at least 24 hours after the last CT injection. Therefore, the possibility that CT induced a transient hypocalcemia of 2–3 hours duration after an S.C. injection [25] cannot be ruled out.

Cancellous bone volume of vehicle-treated control rats remained relatively constant over the entire study (Fig. 1A). In contrast, cancellous bone mass in vehicle-treated OVX rats exhibited a marked decrease of approximately 75% compared with that of baseline control rats, which was at least a factor of 4 less than that of vehicle-treated control rats throughout the study. CT treatment provided partial protection against cancellous bone loss in OVX rats as the mean values for this group were significantly greater than those of vehicle-treated OVX rats, but significantly less than those of vehicle-treated control rats. Within 30 days after withdrawal of CT, cancellous bone volume declined by 50% and rapidly reached the osteopenic level of vehicle-treated OVX rats.

Osteoclast surface of vehicle-treated OVX rats increased significantly relative to that of vehicle-treated control rats at all time points within 90 days after surgery (Fig. 1B). However, osteoclast surface of CT-treated OVX rats was decreased compared with that of vehicle-treated OVX rats, although this decrease reached statistical significance only at 60 days. During the withdrawal period, osteoclast surface in CT-treated OVX rats changed little compared with earlier time points. But due to age-related declines in this variable in vehicle-treated control and OVX groups during the later stages of the study, osteoclast surface of OVX rats previously treated with CT was significantly greater than that of both vehicle-treated groups.

In vehicle-treated control rats, osteoblast surface exhibited a trend for an age-related decrease throughout the study (Fig. 1C). This variable was significantly increased in vehicle-treated OVX rats up to 90 days postsurgery. In contrast, osteoblast surface in CT-treated OVX rats was significantly decreased over the entire treatment period compared with that of vehicle-treated OVX rats. Soon after CT withdrawal, osteoblast surface increased markedly, then subsided somewhat, but was still significantly higher than that of both vehicle-treated groups.

The effects of ovariectomy and CT treatment and withdrawal on mineralizing surface (data not shown) were similar to those described above for osteoblast surface. Mineral apposition rate (data not shown) was significantly increased in vehicle-treated OVX rats compared with vehicle-treated control rats at 30, 60, and 90 days postsurgery. Afterwards, this variable declined in vehicle-treated OVX rats toward control levels. CT treatment of OVX rats significantly decreased mineral apposition rate to the level of vehicle-treated control rats at 60 and 90 days. During the withdrawal period, this variable increased somewhat in OVX rats previously treated with CT so that there were no longer any significant differences between this group and vehicle-treated OVX rats.

Bone formation rate decreased with age in vehicle-treated control rats (Fig. 1D). Compared with this group, bone formation rate was significantly increased in the vehicle-treated OVX group at each time point. At 60 and 90 days, bone formation rate was significantly decreased in CT-treated OVX rats to near the level of vehicle-treated

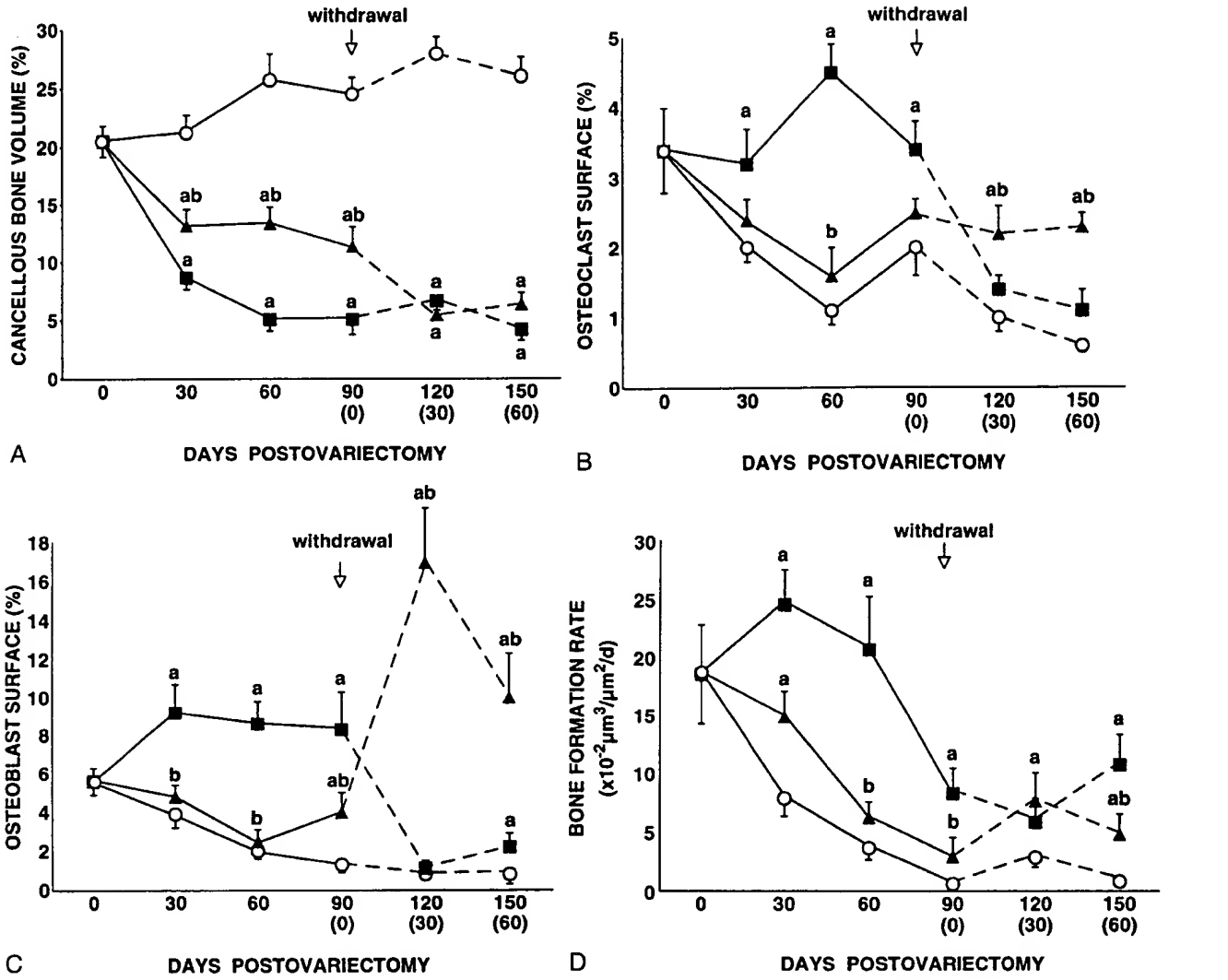

Fig. 1. Cancellous bone volume **(A)**, osteoclast surface **(B)**, osteoblast surface **(C)**, and bone formation rate **(D)** in the proximal tibial metaphysis of the three groups of rats are plotted as a function of time postovariectomy. All rats were treated with vehicle (VEH) or calcitonin (CT) for the first 90 days (solid lines), followed by withdrawal of treatment for the duration of the study (broken lines). Numbers in parentheses on the x-axis are times after withdrawal of treatment. Each data point is the mean ± SE of 6–10 animals. (○) CON + VEH; (■) OVX + VEH; (▲) OVX + CT. [a]Significantly different from vehicle-treated control group ($P < 0.05$). [b]Significantly different from vehicle-treated OVX group ($P < 0.05$).

control rats. Withdrawal of CT treatment resulted in an increase in bone formation rate to the level of vehicle-treated OVX rats at 120 days (30 days of withdrawal). Afterwards, bone formation rate in OVX rats previously treated with CT was significantly less than that of vehicle-treated OVX rats, but significantly greater than that of vehicle-treated control rats.

Discussion

The present study shows that long-term treatment of OVX rats with CT depresses bone turnover and partially prevents the development of cancellous osteopenia. Furthermore, soon after withdrawal of CT treatment, OVX rats exhibit increased bone turnover and rapid loss of cancellous bone mass. These results from an animal model of estrogen depletion are consistent with clinical trials of the bone protective effect of CT as well as the loss of this protective effect after CT withdrawal in postmenopausal women [14]. Despite this consistency, one must be cautious in extrapolating between data obtained from animal studies and clinical trials. For example, the rate of bone loss after CT withdrawal in women is undoubtedly affected by factors not addressed in the current animal study such as age and stage of menopause, severity of osteopenia, baseline rate of bone turnover, and dose and duration of CT treatment.

It is interesting to compare the bone protective effect of CT in OVX rats with that of other antiresorptive agents such as estrogen and bisphosphonates. In our prior studies with OVX rats of nearly identical ages at the beginning of treatment, estrogen and bisphosphonates provided complete protection against cancellous bone loss [26, 27] whereas in the current study, CT had only a partial bone protective effect. Since all three treatments were found to depress bone formation as well as bone resorption, the observed difference in the efficacy of the antiresorptive agents cannot be explained on the basis of different effects on bone formation.

Bonucci et al. [18] also detected only a partial bone protective effect with a similar dose of CT in OVX rats. Although it may be possible that a higher dose of CT may have had a stronger bone protective effect, such a dose would probably also have had a marked anorectic effect [28, 29]. Even at the dose used in the current study (16 U/kg), which is much higher than the human dose (1–3 U/kg), the CT-treated OVX rats weighed significantly less than the vehicle-treated OVX rats. Therefore, a higher dose of CT would have undoubtedly introduced weight loss as a complicating variable for data interpretation. In any case, since the skeletal response to antiresorptive agents is similar in estrogen-deplete rats and women, our findings suggest that CT may not be as effective as estrogen or bisphosphonates for the prevention of early postmenopausal bone loss. Similarly, in patients with established osteoporosis, Adami et al. [30] found that the bisphosphonate alendronate was much more effective than CT in depressing bone turnover and modestly increasing bone mass.

Trends for decreased bone volume and increased cellular indices of bone turnover were observed in OVX rats at 90 days of CT treatment compared with 60 days of treatment with the hormone. Although nonsignificant, these trends suggest that the skeletal response to CT may be declining with length of treatment. This phenomenon has been reported in some clinical trials involving long-term CT treatment of postmenopausal osteoporotic patients [31, 32]. "Escape" from the skeletal effects of CT has been attributed to progressive down-regulation of bone binding sites for the hormone [33]. It has also been suggested that skeletal resistance to CT may develop as a consequence of antibody formation [34, 35]. However, evidence to the contrary [8] makes the significance of the latter mechanism unclear.

Overgaard et al. [14] found that, after withdrawal of CT treatment, postmenopausal women exhibited bone loss associated with increased bone turnover. Pazzaglia et al. [36] also showed that bone resorption increased 20–40 days after withdrawal of CT treatment in intact male rats. Our results in OVX rats are consistent with these findings. The rapid bone changes after withdrawal of CT may be interpreted as evidence for its short biological half-life. It is noteworthy that the rate of bone loss induced by CT withdrawal was similar to that of vehicle-treated OVX rats for the first 30 days postovariectomy, and that both periods of bone loss were associated with increased bone turnover. These findings, which are similar to the skeletal effects of estrogen withdrawal in OVX rats [21], suggest that early postmenopausal women who are withdrawn from prophylactic CT treatment may be at high risk for subsequent bone loss.

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DES inhibits both BF & BR.

Mechanism of Action of Estrogen on Cancellous Bone Balance in Tibiae of Ovariectomized Growing Rats: Inhibition of Indices of Formation and Resorption

RUSSELL T. TURNER, GLENDA L. EVANS, and GLENN K. WAKLEY

ABSTRACT

Ovariectomy results in cancellous osteopenia in rat long bones, a condition that is prevented by treatment with estrogens. The purpose of these studies was to clarify the effects of estrogen on cancellous bone turnover using dynamic bone histomorphometry. Treatment of ovariectomized rats with diethylstilbestrol (DES) reduced the mineral apposition rate, double-label perimeter, osteoblast number, and osteoclast number, suggesting that the hormone had inhibitory effects on bone formation as well as bone resorption. However, we could not estimate the bone formation rate because of rapid resorption of tetracycline-labeled bone in the ovariectomized rat. The magnitude of loss was documented by a time course study: 58% of the tetracycline initially incorporated into the secondary spongiosa of the tibial metaphysis was resorbed after 11 days and 89% was resorbed after 22 days. Similarly, cancellous bone area was decreased by 67% after 11 days and by 88% after 22 days. Administration of either DES or tamoxifen (TAM) dramatically reduced resorption of tetracycline as well as the decrease in cancellous bone area. These results demonstrate that (1) estrogen prevents osteopenia in ovariectomized (OVX) rats, in part by inhibiting bone turnover, (2) TAM is an estrogen agonist on bone resorption, and (3) resorption of tetracycline-labeled bone leads to serious underestimation of the bone formation rate in OVX rats.

INTRODUCTION

There is pronounced sexual dimorphism in skeletal architecture and mass in rats and humans.[1-3] Gonadal hormones appear to be responsible for certain aspects of this sexual dimorphism in both species, including the higher peak bone mass found in males.[3-6] In rats, gonadectomy obliterates the sex difference in radial growth of long bones, but this difference is reestablished after administration of estrogens to ovariectomized (OVX) females and androgens to orchiectomized males.[5,6]

Sex steroids, especially estrogen, have profound effects on longitudinal growth and cancellous bone turnover. High doses of estrogen inhibit longitudinal growth of long bones in girls,[7] as well as in growing female rats.[8] Ovariectomy in rats is believed to result in increases in bone resorption, and to a lesser extent bone formation, leading to an overall loss of cancellous bone.[9,10] Similar mechanisms may be responsible for the net loss of cancellous bone in OVX and postmenopausal women.[11,12] The increase in bone formation could be due to estrogen deficiency per se or, alternatively, secondary to increased bone resorption. Whatever the mechanism, these latter changes are clinically important because they are partly responsible for the marked increase in fracture risk in postmenopausal women.[3]

Some studies suggest that estrogen replacement therapy prevents osteopenia in OVX and postmenopausal women[11,12] and OVX rats[10,13] by decreasing bone resorption, and to a lesser extent bone formation, with an overall improvement in bone balance. Other investigators have reported that estrogen treatment increases bone measurements related to bone formation in OVX[14] and intact[15] rats. However, the results of all the studies thus

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far[10,13-15] are equivocal because there have been no direct measurements of bone resorption and changes in longitudinal growth, endochondral ossification, and bone resorption may have confounded estimation of bone formation. To help clarify the actions of estrogen on bone balance, we developed new methods to estimate growth-independent changes in bone resorption and bone formation in the secondary spongiosa of the tibial metaphysis. In the present study, we applied these techniques to determine the effects of the nonsteroidal synthetic estrogen diethylstilbestrol (DES) and the partial estrogen agonist tamoxifen (TAM) on sequential bone turnover.

MATERIALS AND METHODS

In experiment 1, a time course was determined for the effects of DES on bone dynamic histomorphometry in the tibial metaphysis. OVX rats 10 weeks old weighing 200 g were divided into four groups of 10 animals. Two of the groups were implanted subcutaneously (SC) with controlled-release pellets containing DES 7 days after OVX. The other two groups were given similar pellets without DES as controls. One control and one DES group of rats were given tetracycline 24 h following pellet implantation (tetracycline hydrochloride; Sigma Chemical Co., St. Louis, MO) by intraperitoneal (IP) injection (20 mg/kg). These same groups were given a second injection of tetracycline 5 days following the first and sacrificed 24 h later with CO_2 gas. The remaining group of DES-treated and control rats were injected with tetracycline 8 and 13 days after the implantation of the pellets and sacrificed on day 14. Following sacrifice the tibiae were excised and processed for histomorphometry.

Experiment 2 was a time course to determine the resorption of tetracycline from cancellous bone in the secondary spongiosa; any resorption of tetracycline in a double-label experiment, such as experiment 1, would lead to underestimation of the bone formation rate. Rats 8 weeks old weighing 170 g (Harlan Sprague-Dawley, Indianapolis, IN) were divided into three groups of six to eight animals. All rats were given tetracycline as a single bolus injection IP (20 mg/kg) 7 days after OVX. Groups of rats were sacrificed 6 h, 11 days, and 22 days after administration of the fluorochrome. Following sacrifice, the tibiae were excised, fixed in 70% ethanol, dehydrated with increasing concentrations of ethanol, embedded mineralized in methyl methacrylate, and sectioned at an indicated thickness of 5 μm for subsequent measurement of static and dynamic bone histomorphometry.

Experiment 3 was designed to determine if DES and TAM alter the resorption of tetracycline incorporated into cancellous bone of OVX rats. Any change in resorption of the fluorochrome would further complicate interpretation of bone formation values calculated using conventional double-label methods. DES was used because this potent, nonsteroidal synthetic estrogen has only a weak affinity for other classes of sex steroid receptors and is not metabolized to other classes of sex steroid. TAM was used because this partial estrogen agonist was shown to prevent the increase in osteoclast number following ovariectomy,[10] as well as measurements related to bone resorption after sciatic neurotomy.[16] Tetracycline was administered to 8-week-old OVX rats weighing 170 g 1 week following surgery. One group of six rats was sacrificed 6 h following administration of the fluorochrome to provide baseline measurements. Hormone replacement treatment was then initiated. Groups of six rats were implanted SC with controlled-release pellets designed to administer a constant release (2.5 mg in 3 weeks) of DES (Innovative Research, Toledo, OH) or, in the case of the controls, drug-free pellets. This experiment was then repeated except that the treated rats were administered controlled-release pellets containing TAM (5 mg in 3 weeks). The drug-treated rats and control animals were sacrificed with CO_2 gas 11 days following administration of the fluorochrome.

Bone histomorphometry

Histomorphometric measurements were performed with a SMI-Microcomp-P.M. semiautomatic image analysis system (Southern Micro Instruments, Inc., Atlanta, GA) consisting of a computer (Compak) coupled to a photomicroscope and image analysis system.[16] In this system, a high-resolution color video camera records the image of the specimen through the microscope and displays the image on a video minitor that registers the movement of a digitizing pen on a graphics tablet. As the pen is moved along the graphics tablet, a tracing appears superimposed on the image of the specimen displayed on the video screen. The region of interest is traced, the line lengths and area bounded by the lines are automatically calculated by the computer, and the values are stored until recalled for analysis.

Cancellous bone measurements were made on unstained sections. A standard sampling site was established in the secondary spongiosa of the metaphyseal region of the proximal tibia 1 mm distal to the tetracycline label that was deposited in the mineralizing cartilage of the epiphyseal growth plate, its center being perpendicular to and on the long axis of the bone.[10] This method differs from conventional methods, which locate the sampling site relative to the growth plate. Our modified method compensates for differences in longitudinal growth between the control and DES-treated rats to ensure that equivalent sampling sites are measured. The site sampled is situated in the secondary spongiosa and extends bilaterally in each section but excludes the cortical edges. A total metaphyseal area of 2.0 mm^2 was sampled for each section.

Double-label dynamic bone histomorphometry

The following measurements were performed on unstained sections under ultraviolet fluorescence (experiment 1):

Cancellous bone perimeter per tissue area (B.Pm/T.Ar, mm/mm^2): total cancellous bone perimeter within the sampling site.

Single-label perimeter per tissue area (sL.Pm/T.Ar, mm/mm^2): total cancellous bone perimeter with one tetra-

TABLE 1. EFFECTS OF DES ON CANCELLOUS BONE HISTOMORPHOMETRY IN THE SECONDARY SPONGIOSA OF OVX RATS[a]

	Week 1 of treatment			*Week 2 of treatment*			*Two-way ANOVA*		
Measurement	*Control*	*DES*	*% Change*	*Control*	*DES*	*% Change*	*Time*	*DES treatment*	*Interaction*[b]
Cancellous bone perimeter, mm/mm²	4.21 ± 0.40	4.98 ± 0.28	18	4.04 ± 0.11	6.72 ± 0.27	67	$p < 0.005$	$p < 0.001$	$p < 0.02$
Single-labeled perimeter, %	16.2 ± 2.9	27.5 ± 3.3	70	17.6 ± 1.6	29.0 ± 4.2	65	NS	$p < 0.005$	NS
Double-labeled perimeter, %	36.6 ± 2.3	35.3 ± 2.8	−4	35.8 ± 2.7	17.4 ± 4.0	−51	$p < 0.01$	$p < 0.03$	$p < 0.02$
Mineral apposition rate, μm/day	2.63 ± 0.10	2.24 ± 0.13	−15	2.26 ± 0.13	1.30 ± 0.10	−42	$p < 0.00015$	$p < 0.0015$	NS
Longitudinal growth rate, μm/day	73.4 ± 5.7	25.6 ± 1.2	−65	71.4 ± 4.9	7.5 ± 0.7	−89	NS	$p < 0.0001$	NS

[a]Values are mean ± SEM (standard error of the mean).
NS, not significant
[b]Interaction is between DES treatment and time.

cycline label. The method does not distinguish between first and second label. The data are expressed as percentage of total cancellous bone perimeter.

Double-label perimeter per tissue area (dL.Pm/T.Ar, mm/mm²): total cancellous bone perimeter with two tetracycline labels. The data are expressed as percentage total cancellous bone perimeter.

Mineral apposition rate (μm/day): mean distance between the two tetracycline labels (Ir.L, μm) divided by the labeling interval of 5 days. This value was the mean determined from 50–100 measurements per section.

Longitudinal growth rate (Lo.Gr.R, μm/day): mean distance from the tetracycline label in the mineralizing cartilage to the tetracycline label in the primary spongiosa measured at five equidistant sites across the growth plate. The mean distance was divided by the labeling interval of 5 days.

Acid phosphatase-stained sections

The following measurements were performed on sections stained for acid phosphatase and counterstained with toluidine blue (experiment 1):

Osteoclast perimeter per tissue perimeter (Oc.Pm/T.Pm, mm/mm): total cancellous bone perimeter with osteoclasts within the standard sampling site. This value is expressed as a percentage.

Osteoclast number per cancellous bone perimeter (cells per mm): number of multinucleated, acid phosphatase-positive cells lining cancellous bone within the sampling site in the secondary spongiosa divided by bone perimeter.

Osteoblast perimeter per bone perimeter (Ob.Pm/B.Pm, mm/mm): total cancellous bone perimeter with osteoblasts within the standard sampling site divided by total perimeter. This value is expressed as a percentage. This method underestimates cell number because some osteoblast profiles are indistinguishable from lining cells.

Resorption of previously incorporated tetracycline

The following measurements were performed on unstained sections under UV fluorescence (experiments 1 and 2):

Label perimeter per tissue area (sL.Pm/T.Ar, mm/mm²): total cancellous bone perimeter with tetracycline label within the standard sampling site. This value is expressed as a perimeter.

Cancellous bone area per tissue are (B.Ar/T.Ar, mm²/mm²): total area of cancellous bone within the sampling site. This value is expressed as a percentage.

RESULTS

The OVX rats increased in body weight by 37% during the 2 weeks following implantation of drug-free pellets (experiment 1). As expected, treatment of OVX rats with either DES or TAM resulted in significantly ($p < 0.01$) smaller increases in weight, with treated rats growing 2% (experiment 1) and 21.3% (experiment 3) less than OVX controls.

The effects of 1 and 2 weeks of DES treatment on dynamic bone measurements in the secondary spongiosa of the cancellous tibial metaphysis (experiment 1) are shown in Table 1. Treatment for 1 week resulted in no change in total cancellous bone perimeter or double-label perimeter, increased the single-labeled perimeter, and decreased both the mineral apposition and longitudinal growth rates compared with OVX rats implanted with drug-free pellets. Treatment for 2 weeks resulted in further decreases in mineral apposition rate and longitudinal growth rate, decreased double-labeled perimeter, and increased single-label perimeter and total cancellous bone perimeter compared with the age-matched drug-free controls.

The effect of 1 week of DES treatment (experiment 1) on cell number is shown in Table 2. DES treatment resulted in decreases in osteoclast perimeter, osteoclast number, and osteoblast perimeter in the secondary spongiosa.

The effects of time on tetracycline-labeled perimeter, cancellous bone area, and longitudinal growth are shown for experiment 2 in Fig. 1. Of the cancellous bone perimeter in the metaphyseal sampling site 20.5 ± 4.2% was labeled at baseline (6 h following administration of tetracycline). The labeled perimeter was decreased by 58% 11 days following administration of the fluorochrome and 89% after 22 days (Fig. 1A). Similarly, there was a 67% reduction in cancellous bone area in the sampling site after 11 days and an 88% reduction after 22 days (Fig. 1B). A linear increase in tibial length due to longitudinal growth was observed (Fig. 1C).

The effects of treatment with DES and TAM on cancellous bone area and tetracycline-labeled perimeter (experiment 3) are shown in Fig. 2. Tetracycline-labeled perimeter was significantly reduced in OVX rats compared to the DES- and TAM-treated OVX animals (Fig. 2A). The DES- and TAM-treated OVX animals did not differ significantly

TABLE 2. EFFECTS OF 1 WEEK OF DES TREATMENT ON BONE CELL MEASUREMENTS IN THE TIBIAL METAPHYSIS OF OVX RATS[a]

Measurement	*Control*	*DES*	*% Change*	p *Value*
Osteoclast perimeter, %	22.5 ± 2.5	5.1 ± 1.4	−77	<0.00015
Osteoclasts, cells per mm	4.7 ± 0.6	1.3 ± 0.3	−72	<0.00025
Osteoblast perimeter, %	23.6 ± 1.8	13.9 ± 2.2	−41	<0.006

[a]Values are mean ± SEM, $N = 7$ or 8.
$p > 0.05$ by unpaired t-test.

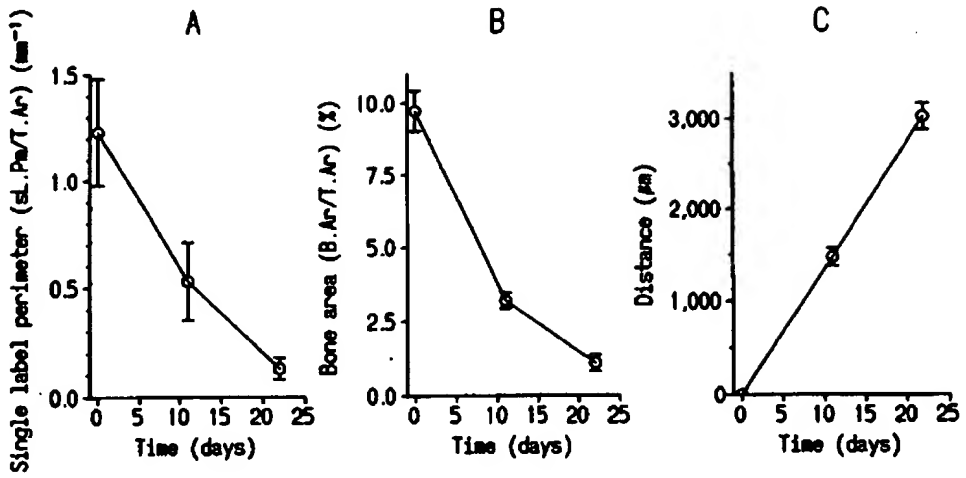

FIG. 1. Effect of time after administration of a single bolus IP injection of tetracycline on (A) tetracycline-labeled perimeter (sL.Pm/T.Ar) and (B) cancellous bone area (B.Ar/T.Ar) in a standard sampling site located in the secondary spongiosa of the tibial metaphysis 1 mm distal to the tetracycline label originally deposited in the zone of mineralizing growth plate cartilage. The effect of time on (C) distance from the label incorporated into the mineralizing growth plate cartilage and the mineralizing growth plate cartilage at sacrifice is also shown. The values are mean ± SEM, $N = 6$–8. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to baseline controls sacrificed 6 h following administration of tetracycline.

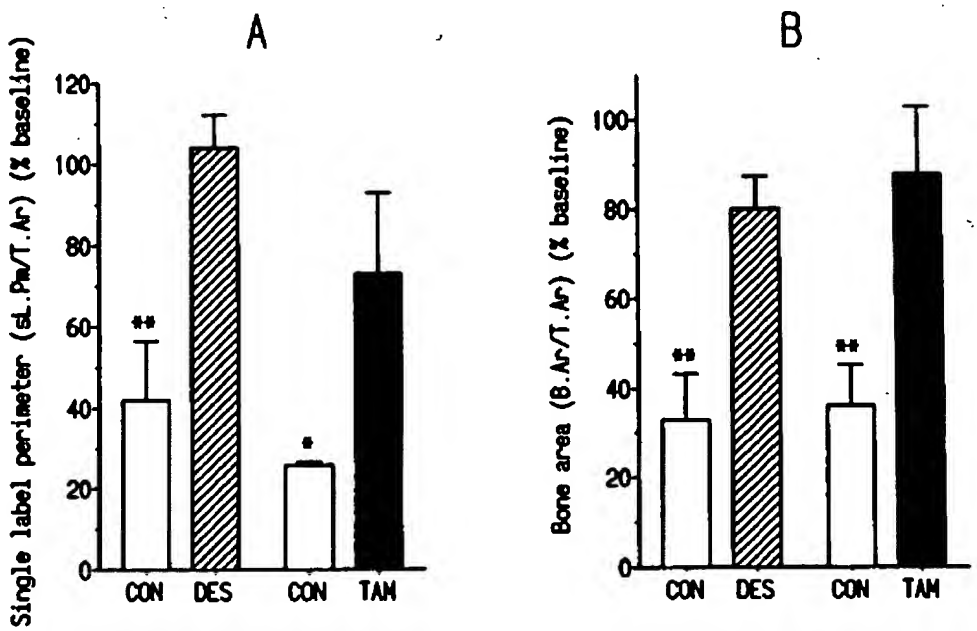

FIG. 2. Effects of DES and TAM on (A) tetracycline-labeled perimeter and (B) cancellous bone area. The rats were sacrificed 11 days following administration of tetracycline and the data expressed as percentage of baseline control. The values are mean ± SEM, $N = 6$–8. $^{*}p < 0.05$, $^{**}p < 0.02$ compared to treated groups.

from their respective baseline values. Cancellous bone area was also significantly reduced in OVX rats compared to the DES- and TAM-treated OVX animals (Fig. 2B). Again, the DES- and TAM-treated OVX animals did not differ significantly from the baseline controls.

DISCUSSION

There is general agreement that bone formation is increased in the tibial metaphysis following OVX. However, the effects of estrogen on bone formation remain controversial. On one hand, Wronski et al.[13] reported that high doses of 17β-estradiol preserve cancellous bone volume in OVX rats by inhibiting bone turnover, and Kalu et al.[17] showed that much smaller doses of 17β-estradiol prevented the increase in mineral apposition rate. In marked contrast, Takano-Yamamoto and Rodan[14] reported that administration of estrogen to femora of OVX rats increased osteoblast number; these authors interpreted the latter results as evidence that estrogen increased bone formation. This conclusion is supported by Tobias et al.,[18] who reported that estrogen increased bone formation in intact female rats.

The results of the present study support the hypothesis that estrogen inhibits bone turnover in OVX rats. In addition to decreasing osteoclast number and inhibiting resorption of previously incorporated tetracycline, DES reduced several measurements related to bone formation: specifically, mineral apposition rate, double-labeled perimeter, and osteoblast number. An important finding in these studies was that as much as 5% of a fluorochrome label incorporated into cancellous bone of an OVX rat was resorbed each day. Resorption of previously incorporated label would also be expected to occur during conventional double-label studies to estimate bone formation and would result in underestimation of the formation rate, especially when the label is administered a long interval before sacrifice. The reliance on labels that are subject to varying rates of bone resorption introduces a potential artefact into calculation of bone formation rate. This artefact could influence interpretation of data obtained using the OVX-estrogen–treated rat model because OVX and estrogens alter bone resorption in opposing directions. Age may also be an important variable. The present studies were carried out in young growing rats; further studies are necessary to establish the effects of bone resorption on fluorochromes incorporated into cancellous bone of mature animals.

Our studies differ from all previous studies in that we adjusted the position of the sampling site to compensate for longitudinal growth and thus limited our measurements to bone undergoing sequential turnover. Comparison of longitudinal growth and mineral apposition rates (Table 1) reveals that longitudinal growth is much more important than remodeling in determining cancellous bone volume in young rats. OVX and estrogen treatment increase and decrease longitudinal bone growth, respectively. Failure to compensate for the growth differences results in nonequivalent sampling of the cancellous bone. As a consequence, observed differences reported for estrogen-treated and control rats may be related to skeletal maturity as well as hormone-induced changes in bone turnover.[18] This complication becomes decreasingly important with age because of greatly reduced longitudinal growth in adult rats.

We characterized the effect of estrogen on retention of tetracycline in cancellous bone in the secondary spongiosa of OVX rats. This method is based on the principle that tetracycline is retained at the site of its initial deposition until that bone undergoes resorption. To achieve equal labeling, the fluorochrome was administered before initiating treatment. Thus, differences in tetracycline-labeled bone at the end of the study must represent an effect of the treatment on net bone resorption. It is assumed that resorption of the bone in which the tetracycline is deposited is representative of bone in the sampling site as a whole, an assumption that appears to be borne out by the parallel time-dependent decreases in bone area and tetracycline-labeled perimeter. Also, the simultaneous decrease in osteoclast number and increases in tetracycline-labeled perimeter and bone volume in DES- and TAM-treated rats is consistent with this interpretation. Since large changes in bone formation have the potential to alter the disappearance of label independently of changes in bone resorption, it is important to evaluate indices of bone formation. In the present study, all measurements related to bone formation were reduced by DES. A decrease in bone formation should result in an overestimation of bone resorption compared to the controls. Thus, the observed inhibitory effect of DES on the disappearance of tetracycline indicates that bone resorption is essentially halted by a high dose of estrogen.

Estrogen treatment reduced osteoclast number and perimeter in OVX rats. This finding is in agreement with previous studies demonstrating an increased osteoclast number in rats following OVX and decreased osteoclast number in OVX rats following treatment with estrogen.[9,10,12,14] TAM was shown in previous studies to prevent cancellous osteopenia following OVX[10] and unilateral sciatic neurotomy.[19] The present results suggest that this protective effect is due, at least in part, to TAM acting as an estrogen agonist on osteoclasts. This conclusion is supported by a study demonstrating that TAM treatment prevents the increases in osteoclast number and size following OVX.[10]

The changes in bone resorption in estrogen-treated rats could result in an increase in double-labeled bone perimeter without there being an increase in bone formation. The magnitude of this label artefact depends upon the age of the rats, labeling schedule, and dose of the hormone and could be responsible for some of the discrepancies in the literature.

Changes in bone resorption do not explain the discrepancy between investigators who report that estrogen increases[15] and decreases[12,17] the mineral apposition rate in cancellous bone. The present study demonstrated a pronounced inhibitory effect of estrogen on the mineral apposition rate in bone at a site located in the secondary spongiosa at the start of treatment. Although a very high dose of DES was administered by continuous-release pellets, we achieved similar results in OVX rats treated with 17β-es-

tradiol at moderate 4 and 8 μg/kg and toxic 4000 μg/kg doses by daily injection, suggesting that neither the rate nor the means of administration of estrogen is critical (data not shown). The present study differs from that of Tobias et al.[15] in that we used OVX rats whereas these investigators used intact animals. However, Wronski et al.[13] did not observe an increase in mineral apposition rate in estrogen-treated rats, suggesting that the difference in results is not due to intact ovaries. Further studies are necessary to clarify these different findings.

The mechanism of action of estrogen on the skeleton is not known. It is clear that the long-term effects of the hormone are influenced by adaptive processes, which may include changes in calcium absorption[20] and mechanical loading.[21] However, there is no compelling evidence that the effects of the hormone are mediated by changes in calcium regulating hormones or other systemic factors.[22] Furthermore, we have shown that estrogen treatment reduces mRNA levels for insulin-like growth factor I in calvarial periosteum within 2 h, suggesting that the hormone has direct effects on cells of bone to regulate their activity.[23]

The results of numerous studies suggest that ovariectomy has tissue-specific effects on bone balance in rat tibiae: there is a net addition of bone to the periosteal surface of the middiaphysis, a small net loss from the endosteal surface, and a large net loss from the trabecular surfaces of the metaphysis.[6,9,10,13,14,22] These differences are due, in part, to regional differences in osteoclasts. There are very few osteoclasts in the periosteum and no evidence that their number is altered by ovariectomy; there are also few osteoclasts on the endosteum, but their number is increased following ovariectomy[22,24]; osteoclasts are prevalent on trabecular surfaces, and their number is increased following OVX.[10,13] Bone formation was increased in OVX rats at the periosteum, with no increase in bone resorption, and bone resorption was increased at the endosteum, with no corresponding consistent increase in bone formation. These observations provide circumstantial evidence against the hypothesis that the increase in bone formation in OVX rats is secondary to changes in bone resorption,[5,6,9,10,13,16,22,24] at least in cortical bone.

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Endocrine and Pharmacological Suppressors of Bone Turnover Protect against Osteopenia in Ovariectomized Rats*

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ABSTRACT. This study was designed to test the hypothesis that endocrine and pharmacological suppressors of bone turnover prevent the development of osteopenia during estrogen deficiency. Sham-operated control and ovariectomized (OVX) rats were treated intermittently with vehicle alone, estrogen, or the diphosphonate compounds etidronate disodium (EHDP) and NE-58095 [2-(3-pyridinyl)2-hydroxyethylidene-1,1-bisphosphonate disodium] for 35 or 70 days after surgery. Their proximal tibiae were processed undecalcified for quantitative bone histomorphometry. Vehicle-treated OVX rats were characterized by decreased cancellous bone volume and 3- to 4-fold increases in osteoblast surface, osteoclast surface, bone formation rate, and bone resorption rate. Treatment of OVX rats with estrogen and NE-58095 provided complete protection against bone loss and significantly depressed all of the above indices of bone turnover. OVX rats treated with EHDP exhibited at least partial protection against bone loss and decreased bone turnover. EHDP induced a mild mineralization defect, as indicated by a prolonged mineralization lag time at the tibial endocortical surface. The new diphosphonate compound NE-58095 did not impair bone mineralization. Our results indicate that endocrine and pharmacological suppressors of bone turnover prevent the development of osteopenia during the early stages of estrogen deficiency. If confirmed by clinical trials in humans, diphosphonate compounds may prove to be an alternative to estrogen for the prevention of postmenopausal bone loss. (*Endocrinology* **125**: 810–816, 1989)

BONE loss during the early stages of estrogen deficiency is clearly associated with increased bone turnover. This phenomenon has been detected in rats (1–6), dogs (7), baboons (8), and humans (9–13). Therefore, it seems logical to hypothesize that suppression of bone turnover in the early estrogen-deficient state would protect against the development of osteopenia. In support of this hypothesis, the mechanism for estrogenic protection against postmenopausal bone loss has been shown to involve depressed bone turnover (10, 14). Estrogen treatment also has a bone protective effect associated with decreased bone turnover in ovariectomized (OVX) rats (3, 5, 15). These findings suggest that other endocrine or pharmacological suppressors of bone turnover may be useful for the prevention of postmenopausal bone loss. In this regard, diphosphonate compounds are of considerable interest due to their well documented suppressive effect on bone turnover (16–18). More specifically, etidronate disodium (EHDP) was recently reported to suppress bone turnover soon after surgical menopause in women (19). However, it remains to be established whether diphosphonate treatment of estrogen-deficient women can maintain bone mass at premenopausal or presurgery levels. Therefore, the purpose of the current study is to compare the therapeutic effects of estrogen and diphosphonate compounds on bone loss in the early estrogen-deficient state. In addition, the skeletal effects of an established diphosphonate compound (EHDP) are compared to those of a new, more potent diphosphonate compound (NE-58095). The OVX rat served as an animal model for this study.

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Materials and Methods

The experimental animals were female Sprague-Dawley rats (Charles River Laboratory, Inc., Wilmington, MA) that were 90 days of age and weighed an average of 240 g at the beginning of the study. All rats were anesthetized with an ip injection of ketamine hydrochloride and xylazine at doses of 50 and 10 mg/kg BW, respectively. Bilateral ovariectomies were performed in half of the rats from a dorsal approach. The remainder were subjected to sham surgeries. The rats were divided into various groups based on treatment with estrogen, diphosphonate compounds, or vehicle alone. Estrogen treatments were performed with 17β-estradiol (Sigma Chemical Co., St. Louis, MO) dis-

solved in a vehicle of 95% corn oil and 5% benzyl alcohol. The diphosphonate compounds (Norwich Eaton Pharmaceuticals, Inc., Norwich, NY) EHDP (ethane-1-hydroxy-1,1-diphosphonate) and NE-58095 [2-(3-pyridinyl)-2-hydroxyethylidene-1,1-bisphosphonate disodium] were dissolved in a saline vehicle. Each of the following groups consisted of 20–24 OVX rats and 20–24 sham-operated control rats.

Vehicle

Half of the OVX and control rats in this group were injected sc with the corn oil-benzyl alcohol vehicle, while the remainder were injected sc with the saline vehicle.

Estrogen

All rats in this group were injected sc with 17β-estradiol 5 days/week at a dose of 10 μg/kg BW.

EHDP (regimen 1)

Each rat in this group was injected sc with EHDP at a dose of 5 mg/kg BW. Treatments were performed daily for a 1-week period, followed by a 3-week period without treatment. This cycle was then repeated for the duration of the study. This regimen will be referred to below as the 1 week on, 3 weeks off regimen.

EHDP (regimen 2)

All rats in this group were injected sc with EHDP twice weekly at a dose of 5 mg/kg BW.

NE-58095

Each rat in this group was injected sc with NE-58095 at a dose of 5 μg/kg BW according to the 1 week on, 3 weeks off regimen.

All of the above treatments were initiated on the first day after surgery. The rats were housed individually at 25 C with a 13-h, 11-h light/dark cycle. Food (Purina rat laboratory chow, Ralston-Purina, St. Louis, MO) was available *ad libitum* to the sham-operated control rats. The food consumption of OVX rats was restricted to that of the control rats (pair-feeding) to minimize the increase in body weight associated with ovariectomy (20). Each rat was injected ip with demeclocycline (Lederle Laboratories, Pearl River, NY) and calcein (Sigma) 14 and 7 days before death, respectively. This regimen resulted in deposition of a double fluorochrome label at bone surfaces that were actively mineralizing throughout the injection period.

Half of the OVX and control rats from each group were killed 35 days after surgery by exsanguination under ketamine/xylazine anesthesia. The remainder were killed 70 days after surgery. In addition, a group of 10 baseline control rats was killed on the day of surgery (day 0). Success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns. The proximal tibiae were stripped of musculature and placed in 10% phosphate-buffered formalin for 24 h. The bone specimens were then dehydrated in ethanol and embedded undecalcified in methyl methacrylate (21). Longitudinal sections (4 μm thick) were cut with an AO Autocut/Jung 1150 microtome and stained according to the Von Kossa method with a tetrachrome counterstain (Polysciences, Inc., Warrington, PA). Bone measurements were performed in the proximal tibial metaphysis with the Bioquant Bone Morphometry Package (R & M Biometrics Corp., Nashville, TN). Cancellous bone areas and surface lengths were traced with a cursor on a Hipad digitizing tablet adjacent to a Nikon Labophot microscope. The light within the cursor can be visualized in the microscopic field when used in conjunction with a camera lucida. Raw data were stored in an Apple IIe microcomputer interfaced to the digitizing tablet. Bone histomorphometric values were then calculated with Bioquant software. Cancellous bone volume (percentage) and osteoclast and osteoblast surfaces as percentages of the total cancellous surface length were measured in this manner. Bone measurements were performed in cancellous bone tissue at distances greater than 1 mm from the growth plate-metaphyseal junction to exclude the primary spongiosa. Additional details of the sample site and data calculations have been reported previously (1, 2). In general, two sections of the proximal tibia with about 40 mm cancellous bone perimeter were sampled in each control animal. The relative lack of bone spicules in OVX rats made it necessary to sample additional sections in these animals. Therefore, surface-based measurements were performed in three or more sections in each OVX rat to approximate the cancellous bone perimeter sampled in control rats.

Fluorochrome-based indices of bone formation were measured in unstained 10-μm thick sections of the proximal tibial metaphysis. The rate of longitudinal bone growth, percentage of cancellous bone surface with a double fluorochrome label (mineralizing surface), and mineral apposition rate were measured with the Bioquant system described above. In addition, bone formation rate (tissue level, total surface referent) was calculated by multiplying mineralizing surface by mineral apposition rate (22). Values for mineral apposition rate were not corrected for obliquity of the plane of section in cancellous bone (22).

Measurement of cancellous bone volume and specific surface in the baseline control animals allowed the calculation of bone balance (22). Bone resorption rate (tissue level, total surface referent) was determined by subtracting bone balance from bone formation rate (22).

The occurrence of osteoid in cancellous bone tissue of most groups of rats was minimal. However, a prominent osteoid seam was present along the endocortical surface of the proximal tibia in all animals. To evaluate the effect of diphosphonate compounds on bone mineralization, osteoid seam width and mineral apposition rate were measured at 10 endocortical sites/animal with the Bioquant system. Mineralization lag time at the tibial endocortical surface was determined by dividing osteoid seam width by mineral apposition rate (23).

Data are expressed as the mean ± SD for each group. Statistical differences among groups were evaluated with the Kruskal-Wallis test (24). $P < 0.05$ was considered significant.

Results

All groups of rats gained substantial weight during the experiment, as their mean body weights increased from

240 g at surgery to 310–360 g 70 days postsurgery. Despite pair feeding, OVX rats in each treatment group weighed about 10% more than their corresponding controls. The exception was the estrogen-treated groups, in which the mean body weights of OVX and control rats were nearly identical. As previously observed (15), estrogen-treated OVX rats weighted about 12% less than vehicle-treated OVX rats (314.8 *vs.* 355.6 g; $P < 0.05$). On the other hand, the mean body weights of diphosphonate-treated OVX rats (340–360 g) were more similar to the mean of vehicle-treated OVX rats (355.6 g), with no significant differences among the groups.

Table 1 lists bone histomorphometric data in cancellous bone tissue of the proximal tibial metaphysis 35 days after surgery. Vehicle-treated OVX rats were characterized by a marked decrease in cancellous bone volume and a 3- to 4-fold increase in osteoblast and osteoclast surfaces relative to those of vehicle-treated control rats. Bone formation rate and bone resorption rate were also increased by at least a factor of 4 in the vehicle-treated OVX animals. Although longitudinal bone growth tended to be increased by ovariectomy, statistical significance was not observed. Mineralization lag time at the tibial endocortical surface was not significantly different in vehicle-treated OVX and control rats.

Estrogen treatment in OVX rats normalized cancellous bone volume and significantly decreased osteoblast surface, osteoclast surface, bone formation rate, and bone resorption rate relative to those in vehicle-treated OVX rats. Longitudinal bone growth was moderately suppressed in estrogen-treated OVX and control rats relative to that in vehicle-treated OVX and control rats. Mineralization lag time was minimally affected by estrogen treatment, with a slight prolongation in estrogen-treated control rats.

OVX rats treated with EHDP according to the 1 week on, 3 weeks off regimen exhibited a normal cancellous bone volume. Osteoblast and osteoclast surfaces were not significantly different in these animals compared to those in vehicle-treated OVX rats, although a strong downward trend was observed in the former parameter. However, EHDP treatment of OVX and control rats markedly depressed both bone formation and bone resorption rate to levels below those in vehicle-treated OVX and control rats. Longitudinal bone growth was also moderately depressed by EHDP treatment. Absolute osteoid volume was 0.3 ± 0.2% in OVX rats treated according to this particular EHDP regimen. This value was significantly greater ($P < 0.05$) than the mean absolute osteoid volume in all other groups (<0.1%). OVX rats treated with the first EHDP regimen also had an increased mineralization lag time relative to vehicle-treated control and OVX rats.

Cancellous bone volume in OVX rats treated with EHDP twice weekly was significantly greater than that in vehicle-treated OVX rats, but significantly less than that in vehicle-treated control rats. Although osteoclast surface was not decreased in OVX rats by EHDP treatment, marked decreases were observed in osteoblast surface, bone formation rate, and bone resorption rate. Longitudinal bone growth was somewhat depressed in EHDP-treated OVX rats relative to that in vehicle-treated OVX rats. Treatment of OVX rats with EHDP

TABLE 1. Bone histomorphometric data in the proximal tibial metaphysis 35 days postsurgery

	Control + VEH	OVX + VEH	Control + EST	OVX + EST	Control + EH1	OVX + EH1	Control + EH2	OVX + EH2	Control + NE-5	OVX + NE-5
Bone vol (%)	23.2 ± 6.8[a]	7.3 ± 3.0[b]	24.5 ± 6.3[a]	21.6 ± 9.8[a]	30.9 ± 4.6[a,b]	21.5 ± 6.5[a]	27.1 ± 7.2[a]	13.6 ± 5.3[a,b]	32.8 ± 5.5[a,b]	34.3 ± 8.2[a,b]
Osteoblast surface (%)	2.2 ± 2.3[a]	8.3 ± 5.2[b]	2.5 ± 1.9[a]	3.1 ± 2.3[a]	0.4 ± 0.3[a,b]	3.4 ± 2.7	1.1 ± 1.0[a]	1.6 ± 1.2[a]	0.1 ± 0.1[a,b]	0.3 ± 0.3[a,b]
Osteoclast surface (%)	1.9 ± 1.1[a]	5.8 ± 2.1[b]	3.2 ± 1.2[a,b]	3.2 ± 1.0[a,b]	2.4 ± 1.4[a]	4.9 ± 2.3[b]	4.3 ± 1.6[b]	7.7 ± 4.3[b]	1.6 ± 0.6[a]	3.0 ± 1.3[a]
Bone formation rate ($\times 10^{-2}$; $\mu m^3/\mu m^3 \cdot day$)	11.1 ± 7.0[a]	46.0 ± 11.4[b]	6.8 ± 4.3[a]	4.8 ± 4.3[a,b]	0.8 ± 1.1[a,b]	4.4 ± 2.4[a,b]	1.6 ± 1.2[a,b]	2.8 ± 1.5[a,b]	0.2 ± 0.3[a,b]	3.3 ± 3.7[a,b]
Bone resorption rate ($\times 10^{-2}$; $\mu m^3/\mu m^3 \cdot day$)	11.0 ± 7.1[a]	47.4 ± 11.5[b]	6.6 ± 4.8[a]	5.1 ± 4.1[a,b]	0.1 ± 0.1[a,b]	4.5 ± 2.0[a,b]	1.2 ± 1.5[a,b]	3.7 ± 1.5[a,b]	0.1 ± 0.3[a,b]	2.2 ± 3.7[a,b]
Longitudinal bone growth (μm/day)	32.7 ± 6.0	39.9 ± 5.4	26.2 ± 4.7[a,b]	22.5 ± 6.8[a,b]	20.4 ± 6.4[a,b]	27.6 ± 4.8[a]	27.0 ± 6.1[a,b]	32.2 ± 5.9[a]	24.2 ± 4.5[a,b]	36.2 ± 9.2
Mineralization lag time (days)	2.3 ± 0.9	3.1 ± 1.2	3.4 ± 1.1[b]	3.6 ± 2.8	3.7 ± 1.4[b]	4.1 ± 0.6[a,b]	2.9 ± 0.7	3.8 ± 0.8[b]	3.3 ± 0.9[b]	3.1 ± 0.7

All values are the mean ± SD. Control and OVX rats were treated with vehicle alone (VEH), 17β-estradiol (EST), EHDP according to the 1 week on, 3 weeks off regimen (EH1), EHDP twice weekly (EH2), or NE-58095 (NE-5).

[a] Significantly different from vehicle-treated OVX value ($P < 0.05$).

[b] Significantly different from vehicle-treated control value ($P < 0.05$).

twice weekly prolonged the mineralization lag time relative to that in vehicle-treated control rats.

OVX rats treated with NE-58095 according to the 1 week on, 3 weeks off regimen were characterized by the following changes relative to vehicle-treated OVX rats: increased cancellous bone volume, decreased osteoblast and osteoclast surface, and decreased bone formation and bone resorption rates. Longitudinal bone growth and mineralization lag time were nearly identical in these groups.

Differences in cancellous bone volume among rats of the various treatment groups are seen in Fig. 1.

Bone histomorphometric data in cancellous bone tissue of the proximal tibial metaphysis 70 days after surgery are listed in Table 2. Bone volume remained markedly decreased in vehicle-treated OVX rats relative to that in vehicle-treated control rats. Although mean osteoblast surface was increased by a factor of 4 in the former animals, statistical significance was not observed. Osteoclast surface remained significantly greater in vehicle-treated OVX rats 70 days postsurgery, but was not as highly elevated as at 35 days. Bone formation rate and bone resorption rate were both markedly increased in vehicle-treated OVX rats. Longitudinal bone growth and mineralization lag time at the tibial endocortical surface were not significantly different in vehicle-treated OVX and control rats.

Treatment of OVX rats with estrogen or diphosphonate compounds intermittently for 70 days had skeletal effects similar to those observed after 35 days of treatment. These effects include normalization of cancellous bone volume, decreases in most indices of bone turnover, and suppression of longitudinal bone growth. As observed at 35 days, treatment of OVX rats with both EHDP regimens did not decrease osteoclast surface, but, rather, decreased the bone resorption rate. In contrast to histomorphometric findings at 35 days, OVX rats treated with EHDP for 70 days according to the 1 week on, 3 weeks off regimen exhibited decreased cancellous bone volume relative to vehicle-treated control rats, but still had a greater cancellous bone volume than vehicle-treated OVX rats. Conversely, cancellous bone volume in OVX rats treated twice weekly with EHDP was no longer significantly less than that in vehicle-treated control rats. Mineralization lag time was not prolonged in EHDP-treated OVX rats at 70 days as it had been at 35 days.

Discussion

The current study demonstrates that endocrine and pharmacological suppressors of bone turnover prevent the development of osteopenia in OVX rats. Previous studies have shown that the mechanism for the bone

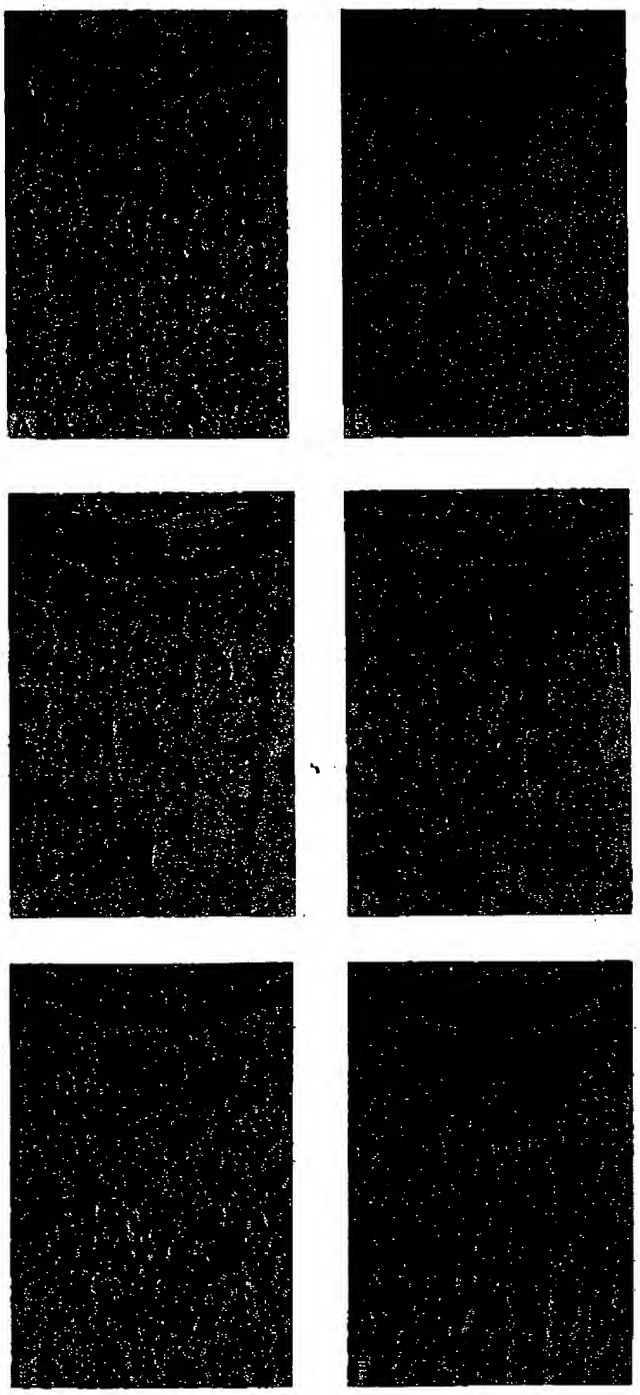

FIG. 1. Proximal tibial metaphyses 35 days postsurgery from control (A) and OVX (B) rats treated with vehicle alone, OVX rats treated with estrogen 5 days/week (C) or EHDP twice weekly (D), and OVX rats treated with EHDP (E) or NE-58095 (F) according to the 1 week on, 3 weeks off regimen. Note the marked loss of cancellous bone spicules in the vehicle-treated OVX animal and the preservation of these spicules in the estrogen-treated OVX animal. Treatment of OVX rats with EHDP twice weekly or with EHDP and NE-58095 according to the 1 week on, 3 weeks off regimen provided at least partial protection

TABLE 2. Bone histomorphometric data in the proximal tibial metaphysis 70 days postsurgery

	Control + VEH	OVX + VEH	Control + EST	OVX + EST	Control + EH1	OVX + EH1	Control + EH2	OVX + EH2	Control + NE-5	OVX + NE-5
Bone vol (%)	28.1 ± 6.2[a]	8.1 ± 3.8[b]	26.7 ± 6.0[a]	24.8 ± 9.9[a]	30.9 ± 7.0[a]	15.4 ± 4.5[a,b]	31.7 ± 6.9[a]	21.4 ± 9.2[a]	39.7 ± 8.5[a,b]	34.5 ± 7.2[a]
Osteoblast surface (%)	1.4 ± 1.2	5.6 ± 4.2	0.7 ± 0.9[a]	0.5 ± 0.6[a,b]	0.1 ± 0.1[a,b]	0.3 ± 0.3[a,b]	0.1 ± 0.1[a,b]	0.3 ± 0.3[a,b]	0.1 ± 0.1[a,b]	0.2 ± 0.2[a,b]
Osteoclast surface (%)	2.8 ± 1.4[a]	4.0 ± 1.1[b]	2.7 ± 1.5[a]	2.2 ± 0.7[a]	2.7 ± 2.0[a]	4.5 ± 1.2[b]	2.7 ± 1.3[a]	4.3 ± 1.5[b]	2.9 ± 1.1	2.6 ± 1.0[a]
Bone formation rate ($\times 10^{-2}$; $\mu m^3/\mu m^2 \cdot day$)	2.9 ± 2.6[a]	25.3 ± 20.4[b]	0.8 ± 0.9[a,b]	1.2 ± 1.6[a,b]	0.1 ± 0.1[a,b]	0.2 ± 0.1[a,b]	0.1 ± 0.1[a,b]	0.7 ± 0.7[a,b]	0.2 ± 0.2[a,b]	0.3 ± 0.3[a,b]
Bone resorption rate ($\times 10^{-2}$; $\mu m^3/\mu m^2 \cdot day$)	2.8 ± 2.8[a]	26.0 ± 20.4[b]	0.6 ± 0.8[a,b]	1.2 ± 1.7[a,b]	0.0 ± 0.0[a,b]	0.5 ± 0.2[a,b]	0.0 ± 0.0[a,b]	0.8 ± 0.9[a,b]	0.0 ± 0.0[a,b]	0.1 ± 0.3[a,b]
Longitudinal bone growth (μm/day)	17.6 ± 3.4	18.8 ± 4.2	9.2 ± 2.7[a,b]	8.7 ± 1.9[a,b]	11.7 ± 2.4[a,b]	14.7 ± 2.6	12.0 ± 3.2[a,b]	12.1 ± 4.0[a,b]	13.3 ± 4.6[a,b]	14.1 ± 3.9[a,b]
Mineralization lag time (days)	3.5 ± 0.8	3.9 ± 1.5	2.0 ± 1.1[a,b]	1.6 ± 0.6[a,b]	3.1 ± 0.8	2.4 ± 0.6[a,b]	2.7 ± 0.6	3.3 ± 1.5	2.6 ± 0.7[a,b]	3.5 ± 0.8

All values are the mean ± SD. See footnotes for Table 1 for a description of the groups.
[a] Significantly different from vehicle-treated OVX value ($P < 0.05$).
[b] Significantly different from vehicle-treated control value ($P < 0.05$).

protective effect of estrogen treatment involves suppression of bone turnover in estrogen-deficient women and rats (3, 10, 14, 15). Our histomorphometric results confirm these findings and further indicate that diphosphonate compounds have similar skeletal effects. EHDP provided at least partial protection against bone loss in OVX rats, whereas treatment of OVX rats with NE-58095 not only prevented bone loss, but increased cancellous bone volume to levels greater than those in vehicle-treated control rats. Both diphosphonate compounds markedly depressed bone turnover. Although diphosphonates have been used in cyclical coherence therapies for potential restoration of lost bone mass in osteoporotic patients (25–27), clinical trials of these compounds for the prevention of bone loss, specifically in the early postmenopausal period, have not yet been reported. Our results indicate that diphosphonate compounds should be considered for additional study as a preventive measure against bone loss soon after surgical or naturally occurring menopause.

Although estrogen and diphosphonate compounds both depressed bone turnover in OVX rats, some subtle differences were detected in their mechanism of action. Estrogen treatment decreased osteoclast number and activity in OVX rats, as indicated by significant declines in osteoclast surface and bone resorption rate relative to those in vehicle-treated OVX rats. EHDP treatment did not decrease osteoclast number, but, rather, inhibited their activity. This finding is consistent with observations by previous investigators in intact rats treated with EHDP (28–30). The skeletal effects of NE-58095 are more similar to those of estrogen in that this new diphosphonate compound depresses osteoclast number as well as activity in OVX rats.

Impaired bone mineralization is a potentially deleterious side-effect of high doses of certain diphosphonate compounds (28–30). In the current study, treatment regimens with EHDP and NE-58095 included a 3-week period without treatment in order to minimize the potential for adverse skeletal side-effects. Nevertheless, EHDP induced a mild mineralization defect in animals treated for 35 days according to the 1 week on, 3 weeks off regimen, as indicated by a prolonged mineralization lag time at the tibial endocortical surface. This parameter returned to normal after 70 days of EHDP treatment according to the same regimen. However, this finding should not be interpreted to mean that the mineralization defect induced by EHDP occurs only during the early stages of treatment. In animals treated with EHDP for 35 days according to the regimen described above, diphosphonate treatment was performed for the final 7 days before death. Conversely, animals treated with EHDP intermittently for 70 days were not administered EHDP during the final week of the study. Therefore, these latter animals may have had sufficient time to recover from an EHDP-induced mineralization defect before death. It is important to note that animals treated with EHDP twice weekly or with NE-58095 according to the 1 week on, 3 weeks off regimen exhibited minimal

against cancellous bone loss. The osteopenic zone evident in the EHDP-treated OVX animal (E) corresponds to the 3 week off period. An osteopenic zone is also present in the OVX rat treated with NE-58095 (F), but it is not as well defined as in the EHDP-treated OVX animal. Von Kossa stain; magnification, ×15.

signs of impaired bone mineralization. The new diphosphonate NE-58095 is particularly impressive in that it provides complete protection against osteopenia in OVX rats without skeletal side-effects at a 1000-fold smaller dose than EHDP.

The proximal tibial metaphysis of OVX rats treated with the first EHDP regimen exhibits a band-like pattern of cancellous bone spicules corresponding to the on and off periods. The most distal band of dense cancellous bone corresponds to the first week of EHDP treatment. The relatively osteopenic zone situated proximal to this band is coincident with the 3-week off period. Finally, the area of dense cancellous bone immediately distal to the growth plate corresponds to the final week of EHDP treatment. OVX rats treated with NE-58095 exhibited a similar metaphyseal band-like pattern, but the osteopenic zone was not as pronounced as in EHDP-treated OVX rats. This band-like pattern of metaphyseal cancellous bone in diphosphonate-treated OVX rats is a consequence of longitudinal bone growth. The new bone spicules left behind by the advancing growth plate during the 3-week off period were protected minimally by diphosphonate treatment. Consequently, the great majority of these bone spicules were presumably resorbed by osteoclasts to create an osteopenic zone. It is important to note that such new bone spicules and the corresponding osteopenic zone would probably not be present in adult animals or humans with closed growth plates and the resultant cessation of longitudinal bone growth.

The moderate suppression of longitudinal bone growth in estrogen-treated OVX rats suggests that the dose of the hormone was supraphysiological. Indices of bone turnover, such as bone formation and resorption rates, were also decreased in estrogen-treated OVX rats relative to those in vehicle-treated control rats. In our previous study (15), estrogen treatment consisted of daily injections at a dose of 10 μg/kg BW. Since this dose and regimen depressed longitudinal bone growth, estrogen treatment was reduced to 5 days/week at the same dose in the current study. Although the suppressive effect of estrogen on longitudinal bone growth was lessened, the persistence of this effect in the current study indicates that estrogen can be used at a lower dose or administered more intermittently to OVX rats to achieve physiological skeletal effects.

Although the skeletal effects of estrogen deficiency and hormone replacement are qualitatively similar in rats and humans (15, 31), caution should be exercised in extrapolating data derived from a growing skeleton to adult bone. For example, longitudinal bone growth in rats is a complicating variable. One should consider whether the suppressive effect of estrogen and EHDP on longitudinal bone growth in OVX rats may have preserved cancellous bone mass. Frost (32) has stated that decreased longitudinal bone growth would result in a lower, rather than greater, cancellous bone volume. In the current study intermittent treatment of OVX rats with NE-58095 for 35 days protected against osteopenia without affecting longitudinal bone growth. Therefore, we consider it unlikely that partial inhibition of longitudinal bone growth by estrogen and EHDP would prevent the development of osteopenia in OVX rats.

In summary, the current study demonstrates that suppression of bone turnover prevents the development of osteopenia during estrogen deficiency. Estrogen and the diphosphonate compounds EHDP and NE-58095 were found to be protective against bone loss in OVX rats, with a similar mechanism of action, *i.e.* suppression of bone turnover. These findings suggest that diphosphonate compounds may prove to be an alternative to estrogen for the maintenance of postmenopausal bone mass. In view of the qualitative similarities between rats and humans in their skeletal responses to estrogen deficiency, hormone replacement, and diphosphonates, the OVX rat may be an appropriate animal model for the initial preclinical evaluation of new therapies designed to prevent postmenopausal bone loss.

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Interleukin-1 Receptor Antagonist Decreases Bone Loss and Bone Resorption in Ovariectomized Rats

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Abstract

Interleukin-1 (IL-1), a cytokine produced by bone marrow cells and bone cells, has been implicated in the pathogenesis of postmenopausal osteoporosis because of its potent stimulatory effects on bone resorption in vitro and in vivo. To investigate whether IL-1 plays a direct causal role in post ovariectomy bone loss, 6-mo-old ovariectomized rats were treated with subcutaneous infusions of IL-1 receptor antagonist (IL-1ra), a specific competitor of IL-1, for 4 wk, beginning either at the time of surgery or 4 wk after ovariectomy. The bone density of the distal femur was measured non invasively by dual-energy X-ray absorptiometry. Bone turnover was assessed by bone histomorphometry and by measuring serum osteocalcin, a marker of bone formation, and the urinary excretion of pyridinoline cross-links, a marker of bone resorption. Ovariectomy caused a rapid increase in bone turnover and a marked decrease in bone density which were blocked by treatment with 17β estradiol. Ovariectomy also increased the production of IL-1 from cultured bone marrow cells. Ovariectomy induced bone loss was significantly decreased by IL-1ra treatment started at the time of ovariectomy and completely blocked by IL-1ra treatment begun 4 wk after ovariectomy. In both studies IL-1ra also decreased bone resorption in a manner similar to estrogen, while it had no effect on bone formation. In contrast, treatment with IL-1ra had no effect on the bone density and the bone turnover of sham-operated rats, indicating that IL-1ra specifically blocked estrogen-dependent bone loss. In conclusion, these data indicate that IL-1, or mediators induced by IL-1, play an important causal role in the mechanism by which ovariectomy induces bone loss in rats, especially following the immediate post ovariectomy period. (*J. Clin. Invest.* 1994. 93:1959–1967.) Key words: interleukin-1 receptor antagonist • osteoporosis • cytokines • estrogen, interleukin-1

Introduction

Postmenopausal osteoporosis is a disorder characterized by a progressive loss of bone tissue which begins after natural or surgical menopause and leads to the occurrence of spontaneous fractures (1). Although the causal role of estrogen deficiency in this condition is well established (2–4), the mechanism by which estrogen prevents bone loss is still conjectural at best. One such mechanism may be a modulatory effect on the secretion of factors that are produced in the bone microenvironment and influence bone remodeling (5). Among them is IL-1, a family of cytokines (6) best known for its involvement in inflammation and wound healing (7, 8), which is also recognized for its effects on bone remodeling. IL-1 stimulates bone resorption in vitro (9, 10) and in vivo (11, 12) and induces hypercalcemia when injected into normal mice (13) by stimulating the activity of mature osteoclasts (14) and the differentiation of osteoclast precursors (15). Studies have also shown that IL-1 inhibits bone formation in vitro (16) and in vivo (17) and induces bone cells to secrete several cytokines such as IL-6 (18), TNFα (15), and M-CSF (19), which regulate the proliferation of osteoclast precursor cells and their differentiation into active osteoclasts.

A role for IL-1 in postmenopausal bone loss is supported by recent studies showing that the expression of IL-1 mRNA is increased in bone cells from postmenopausal women (20) and by previous observations from us (21, 22) and others (23, 24) demonstrating that both natural and surgical menopause are associated with an increased production of IL-1 bioactivity from cultured monocytes that is blocked by estrogen replacement. However, the relevance of these findings with respect to the mechanism by which estrogen prevents bone loss has been difficult to elucidate, because estrogen regulates the production of several cytokines with overlapping effects on bone remodeling (25, 26).

Recently, a specific human IL-1 receptor antagonist (IL-1ra)[1] has been cloned and expressed in *Escherichia coli* with production of a recombinant 17-kD protein which shares 26% sequence homology with IL-1β (27, 28). IL-1ra binds to IL-1 receptors and competes with both IL-1α and IL-1β without detectable IL-1 agonistic effects (29, 30). Human IL-1ra has provided a tool for blocking the effects of IL-1 in several rat cell types (31, 32) including bone cells (33) and to investigate the role of IL-1 in many diseases (34).

We have now assessed the effects of IL-1ra on bone mineral density (BMD) and bone turnover in ovariectomized rats to investigate the role of IL-1 in the bone loss caused by estrogen deficiency.

Methods

Study protocol. Two experiments were conducted in 6-mo-old nulliparous rats (Harlan Sprague-Dawley, Inc., Madison, WI) subjected to

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1. *Abbreviations used in this paper:* BMD, bone mineral density; IL-1ra, IL-1 receptor antagonist; TBV, trabecular bone volume.

either dorsal ovariectomy or sham operation under general anesthesia. In each study ovariectomized animals were treated for 4 wk with continuous subcutaneous infusions of either IL-1ra (Synergen Inc., Boulder, CO), IL-1ra vehicle, BSA, or 10 μg/kg body wt per d 17β estradiol. Sham-operated animals were treated for 4 wk with IL-1ra, BSA, or control vehicle. In study 1, treatments were started at the time of surgery. In this study ovariectomized rats were treated with either 0.4, 2, or 10 mg/kg body wt per d of IL-1ra or 2 mg/kg body wt per d, BSA. Sham-operated rats were treated with 10 mg/kg body wt per d of IL-1ra or 10 mg/kg body wt per d BSA. In study 2, treatments were started 4 wk after surgery and the doses of IL-1ra and BSA were 2.0 mg/kg body wt per d for both the ovariectomized and the sham-operated rats. All treatments were delivered by implanting Alzet osmotic pumps (Alza Inc., Palo Alto, CA) in a dorsal subcutaneous pocket at time 0 and 2 wk of treatment. Alzet 2002 pumps were used to deliver IL-1ra at the dose of 0.4 and 2.0 mg/kg body wt per d, BSA at 2.0 mg/kg body wt per d, 17β-estradiol, or IL-1ra suspension vehicle. Alzet 2ML2 pumps were used to deliver IL-1ra and BSA at 10 mg/kg body wt per d. None of the rats exhibited evidence of infectious disease, impaired growth, immunosuppression, or other side effects caused by the IL-1ra treatment. In a 10-d observation period preceding surgery, the ad lib. food consumption (regular rat Chow, Ralston Purina Co., St. Louis, MO) was 17 g/d. After surgery all rats were fed this amount of food in order to prevent unequal weight changes in the ovariectomized and sham-operated rats. Both at baseline and at the end of each study there were no differences in body weight between the ovariectomized and the sham-operated animals, nor between treatment groups (data not shown).

This protocol was approved by the Animal Care and Use Committee of the Jewish Hospital of St. Louis.

Bone density measurements. The BMD of the distal femur, an area rich in trabecular bone, was measured noninvasively in anesthetized rats by dual-energy X-ray absorptiometry (35) with a model QDR-1000 densitometer (Hologic Inc., Waltham, MA) using a dedicated software for small animal measurements and a 2-mm collimator. This technique provides an integrated measure of both cortical and trabecular bone. In independent studies (35) the precision and the accuracy in vivo of this technique were determined by performing multiple measurements of the same rat and comparing BMD values with direct ash weight (cortical + trabecular bone) measurements of the femur. In these studies the precision and the accuracy in vivo of this technique were 1.2% and 89.0%, respectively.

Bone histomorphometry. Quantitative bone histomorphometric analysis of the metaphysis of the distal femur and of the tibial diaphysis was carried out in specimens obtained at the end of experiment 2, according to the methods of Baron et al. (36) and Turner et al. (37), respectively. Specimens obtained from an additional group of untreated rats killed 4 wk after ovariectomy were also analyzed. At 9 and 2 d before the rats were killed they were injected i.p. with 20 mg/kg body wt oxytetracycline hydrochloride (Pfizer Inc., Brooklyn, NY). The bone specimens were defleshed, fixed in Millonig's fixative, dehydrated in acetone, and embedded undecalcified in methyl methacrylate. Longitudinal sections (4 μm thick) at a depth of one third to one half the thickness of each bone were prepared from the distal femur and stained with a modified Masson-Goldner trichrome. The region examined in the femur was that extending from 1.0 to 1.9 mm from the epiphyseal growth plate and 250 μm from the endocortical surface. Trabecular bone volume (TBV), osteoclast surface (the percentage of bone surface covered by osteoclasts), osteoclast number per millimeter of bone surface, osteoid surface (the percentage of surface covered by osteoid), and osteoid thickness were measured in the stained sections. Dynamic (tetracycline-based) indices of bone formation were then measured on unstained sections as described (38).

For cortical bone analysis, cross sections 200 μm thick were cut from the tibial diaphysis 15 mm from the proximal end using an Isomet low speed saw with a diamond wafer blade. The sections were ground to a thickness of 20–25 μm and mounted unstained on glass slides for analysis of the percent labeled endocortical and periosteal surfaces under ultraviolet light (38). Under transmitted light the same sections were used to quantify marrow area (the area within the endosteal surface of the specimen), cross-sectional bone area (the area within the periosteal surface of the specimen), cortical bone area (the area determined by subtracting marrow area from the cross-sectional area), and cortical thickness. All measurements were performed using a Bioquant Morphometry System (R&M Biometrics, Inc., Nashville, TN).

Pyridinoline crosslinks assay. The urinary excretion of pyridinoline crosslinks, a marker of bone resorption (39, 40), was measured 2 and 4 wk after surgery in urine samples collected between 2:00 and 6:00 p.m. using an ELISA kit developed by Metra Biosystems (Palo Alto, CA) (41). Briefly, a 10-μl urine sample and 150 μl of rabbit antipyridinoline antiserum were added to a pyridinoline-coated microplate and incubated overnight. After the plates were washed with PBS, 150 μl of goat anti-rabbit IgG alkaline phosphatase conjugate was added to each well. The unbound conjugate was then removed by washing and the enzyme activity measured photometrically by adding an enzyme substrate and using a microplate reader at 405 nM. Results were expressed as nanomoles per millimole of urinary creatinine, as measured by a standard colorimetric technique. The intra- and the interassay variation of this method are < 9% and < 15%, respectively (42).

Osteocalcin assay. Serum osteocalcin, a marker of bone formation (43), was measured 2 and 4 wk after surgery with a previously described radioimmunoassay method which makes use of an antibody highly specific for rat osteocalcin (44). The sensitivity of this assay is 10 pg/ml. All reagents were purchased from Biomedical Technologies Inc. (Stoughton, MA).

IL-1ra assay. Serum levels of IL-1ra were measured at 2 and 4 wk of treatment using a specific ELISA recently described (45). The sensitivity of this assay is 8 pg/ml.

Cells cultures and IL-1 assay. To investigate the effect of ovariectomy on the production of IL-1 from bone marrow cells, additional groups of untreated 6-mo-old rats were subjected to ovariectomy or sham operation as described above and killed 2 and 8 wk after surgery. At sacrifice, femora were removed and dissected free of adherent tissue, the bone ends were cut across the ephiphyses and the bone marrow cavity flushed with RPMI 1640 tissue culture medium supplemented with 10 U/ml heparin and 1 μg/ml DNAase. The bone marrow was fractionated on Ficoll Hypaque to prepare mononuclear cell cultures as described (21, 22). The bone marrow mononuclear cells were seeded at 5×10^6 cells/ml and cultured for 24 h with or without the addition of 100 pg/ml LPS. IL-1 bioactivity was measured in the 24-h culture media of the bone marrow mononuclear cells by assessing the increment in mitogen-induced proliferation of the helper T cell D10.G4.1 (D10 cells) as previously described (21, 22). D10 cell proliferation was converted to arbitrary units per milliliter of IL-1 activity by performing a log-logit transformation of the serial dilution curves and determining the dilution of the test sample that yielded a value corresponding to 50% of the standard IL-1 maximum activity. The standard IL-1 activity was arbitrarily set at 100 U/ml. The nature of the assayed material was confirmed as IL-1 by demonstrating inhibition of the conditioned medium effect on the D10 cell proliferation in the presence of 50 ng/ml IL-1ra.

Assessment of serum neutralization activity. The presence of biologically active IL-1ra in the serum of IL-1ra–treated rats was determined by assessing the serum obtained from ovariectomized rats at the end of the 4-wk-long IL-1ra treatment against IL-1β augmentation of mitogen-induced proliferation of D10 cells. Rat sera (12.5 μl) or IL-1ra (2.5 ng) were serially diluted (1:2) and added to D10 cells seeded in 96-well plates. Recombinant human IL-1β (7.5 pg) was then added to each well. This concentration of IL-1β was selected because it induces 50% maximal augmentation in the D10 cell proliferation assay. At the end of a 3-d culture period the D10 cell proliferation was measured as described above. Results were expressed as percent inhibition of D10 cell proliferation.

Statistical analysis. The effect of surgery and treatment on BMD was assessed by using analysis of variance for repeated measures. Subsequent multiple comparison tests were performed by using the Fisher protected LSD test. Group mean values were compared by two-tailed

Table I. Effect of Ovariectomy and Sham Operation on the Secretion of IL-1 Bioactivity from Bone Marrow Mononuclear Cells

	2 weeks after surgery		8 weeks after surgery	
	Sham operated (n = 7)	Ovx (n = 7)	Sham operated (n = 7)	Ovx (n = 7)
			U/ml	
No LPS No IL-1ra	2.3±0.4	24.5±3.1*	3.2±0.3	42.7±4.3*
No LPS IL-1ra (50 ng/ml)	0.01±0.01‡	0.01±0.01‡	0.01±0.01‡	0.01±0.01‡
LPS (100 pg/ml) No IL-1ra	11.1±1.3	68.0±10.5*	5.2±0.4	93.5±10.4§
LPS (100 pg/ml) + IL-1ra (50 ng/ml)	0.01±0.01‡	0.01±0.01‡	0.01±0.01‡	0.01±0.01‡

6-mo-old untreated rats were subjected to either ovariectomy (Ovx) or sham operation. 2 or 8 weeks after surgery bone marrow mononuclear cells were isolated as described in Methods and cultured with and without LPS for 24 h. IL-1 bioactivity was measured in the culture medium with the D10 cell bioassay with and without 50 ng/ml recombinant IL-1ra, as described in Methods. The nature of the assayed material was confirmed as IL-1 by demonstrating inhibition of the conditioned medium effect on the D10 cell proliferation in the presence of recombinant IL-1ra. Values are ±SEM. * $P < 0.05$ and § $P < 0.01$ compared to the corresponding group of sham-operated rats. ‡ $P < 0.01$ compared to all other groups.

Student's t test, or one-way analysis of variance and Fisher protected LSD test, as appropriate.

Results

Ovariectomy increases the secretion of IL-1 from cultured bone marrow cells. Mononuclear cells cultured for 24 h in polystyrene plates with ordinary tissue culture media (which contains small amounts of LPS) express IL-1 mRNA and secrete small quantities of IL-1 (46, 47). In accordance with these published data, bone marrow cells from untreated sham-operated rats were found to secrete measurable amounts of IL-1 bioactivity into the 24-h culture medium (Table I). When further stimulated with the addition of 100 pg/ml LPS, bone marrow cells secreted higher amounts of IL-1 bioactivity which was neutralized by the addition of IL-1ra to the assay system. 2 and 8 wk after surgery bone marrow mononuclear cells from ovariectomized rats produced higher amounts of IL-1 bioactivity than the corresponding cells obtained from sham-operated animals rats (Table I).

Serum from IL-1ra-treated rats neutralizes IL-1β in vitro. IL-1ra, as measured by a specific ELISA, was not detectable in the serum of rats treated either with vehicle or estrogen. In contrast, rats treated with 2 mg/kg body wt per d IL-1ra had serum IL-1ra levels of 160.6±8.6 ng/ml at 2 wk of treatment and 213.4±14.9 ng/ml at 4 wk. When tested in a proliferation neutralization assay, the serum of rats treated with IL-1ra for 4 wk inhibited IL-1β-induced augmentation of D10 cells proliferation in a manner similar to fresh recombinant IL-1ra (Fig. 1). In these experiments parallel and dose-responsive inhibition curves of IL-1-induced augmentation of D10 cell proliferation were obtained with IL-1ra and serum from IL-1ra- treated rats. The sample dilution containing either 3 μl of serum (0.64 ng of IL-1ra by ELISA) or 0.5 ng of fresh IL-1ra per well inhibited about 50% of the IL-1β-induced augmentation of proliferation.

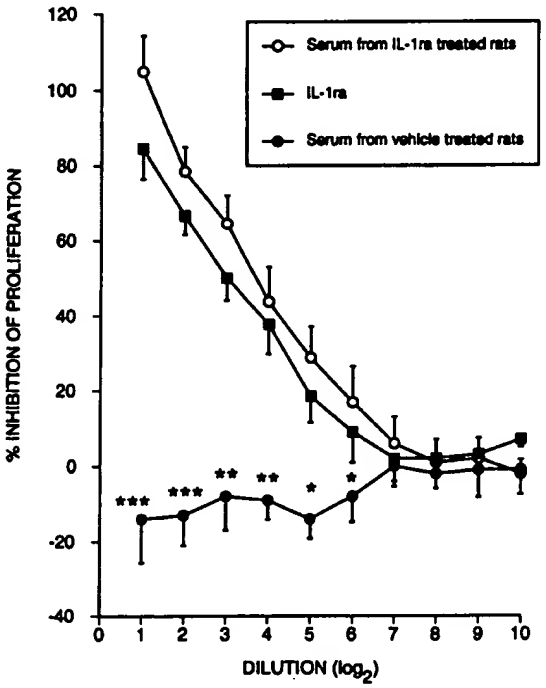

Figure 1. Effect of serum from IL-1ra–treated rats on IL-1β–induced augmentation of D10 cell proliferation (mean±SEM). Sera (12.5 μl) obtained at the end of the treatment period from 14 ovariectomized rats treated with either IL-1ra (n = 7) or vehicle (n = 7) or fresh IL-1ra (2.5 ng) (n = 4) were serially diluted (1:2) and added to D10 cells seeded in triplicate in 96-well plates. Recombinant human IL-1β (7.5 pg) was then added to each well. D10 cell proliferation was measured after a 3-d incubation, as described in Methods. Results (mean of three experiments) are expressed as percent inhibition of D10 cell proliferation. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0001$ compared to the other two groups.

These amounts of IL-1ra were ~ 85 and 67 times the concentration of IL-1β used to stimulate the D10 cells, respectively. Serum from vehicle-treated rats had no effect on IL-1β-induced augmentation of proliferation. These findings demonstrate that a 4-wk treatment with human IL-1ra did not induce the formation of rat anti-human IL-1ra antibodies in an amount capable of blocking the biological effects of IL-1ra.

Effect of IL-1ra treatment on BMD. Fig. 2 shows the effect of IL-1ra treatment on BMD in ovariectomized rats. During the treatment phase of study 1 (Fig. 2 *A*) BMD decreased rapidly in BSA or vehicle-treated ovariectomized rats whereas no significant changes in BMD occurred in groups treated with 17β-estradiol. In this study rats were treated with three doses of IL-1ra. The lowest dose of IL-1ra (0.4 mg/kg body wt per d) was ineffective, as the changes in BMD in this group and in the control groups were similar. In contrast, 2 and 10 mg/kg body wt per d of IL-1ra were both effective, as the bone loss in these groups was lower ($P < 0.05$) than in either the vehicle or the BSA groups. Interestingly, the response to IL-1ra was more pronounced in the second 2 wk of treatment than in the first 2 wk. In fact, the change in BMD between the second and the fourth week of IL-1ra treatment was not significant.

In the pretreatment phase of study 2 (Fig. 2 *B*) BMD decreased significantly in all groups of ovariectomized rats. During the treatment phase of this study BMD continued to decrease rapidly in rats treated with BSA or vehicle alone whereas no additional bone loss occurred in the estrogen-treated rats. In study 2, IL-1ra completely arrested ovariectomy-induced bone loss. As a result, during the treatment period of this study there was no significant bone loss in either the IL-1ra or the estrogen treated rats ($P < 0.05$ compared to the vehicle or the BSA groups for both the estrogen and IL-1ra groups) and the BMD values at the end of the study were similar in the IL-1ra and the estrogen groups.

Table II shows the effect of IL-1ra treatment in sham-operated animals. In each of the two studies a small, nonsignificant decrease in BMD was observed over time in all groups of sham-operated animals. These changes were similar in the vehicle, the BSA and the IL-1ra groups, a finding indicating that IL-1ra specifically prevents the bone loss associated with ovariectomy.

At the end of each study the weight of the uterus was lower in ovariectomized rats treated with vehicle or IL-1ra than in ovariectomized rats treated with estrogen or sham-operated rats treated with either vehicle or IL-1ra. In contrast, no difference in uterine weight was found between estrogen treated ovariectomized rats and sham-operated rats (data not shown). These findings demonstrate the efficacy of the ovariectomy and suggest that IL-1ra has no estrogen-like effects.

Effect of IL-1ra treatment on bone histomorphometry. In a preliminary study we determined that in rats of 6 mo of age, 8 wk are required for ovariectomy to induce a significant decrease in TBV, as assessed by histomorphometric analysis of distal femur metaphysis. For this reason, bone histomorphometry was used to analyze the effects of IL-1ra on bone volume and bone turnover in the femur metaphysis and tibial diaphysis of rats treated between weeks 4 and 8 after surgery (experiment 2). Specimens from an additional group of untreated rats killed 4 wk after ovariectomy were also analyzed and used as additional (baseline) controls. Moreover, since bone densitometry showed that ovariectomy causes a similar bone loss in rats treated with vehicle and BSA, quantification of histomorphometric indices was not performed in rats treated with BSA.

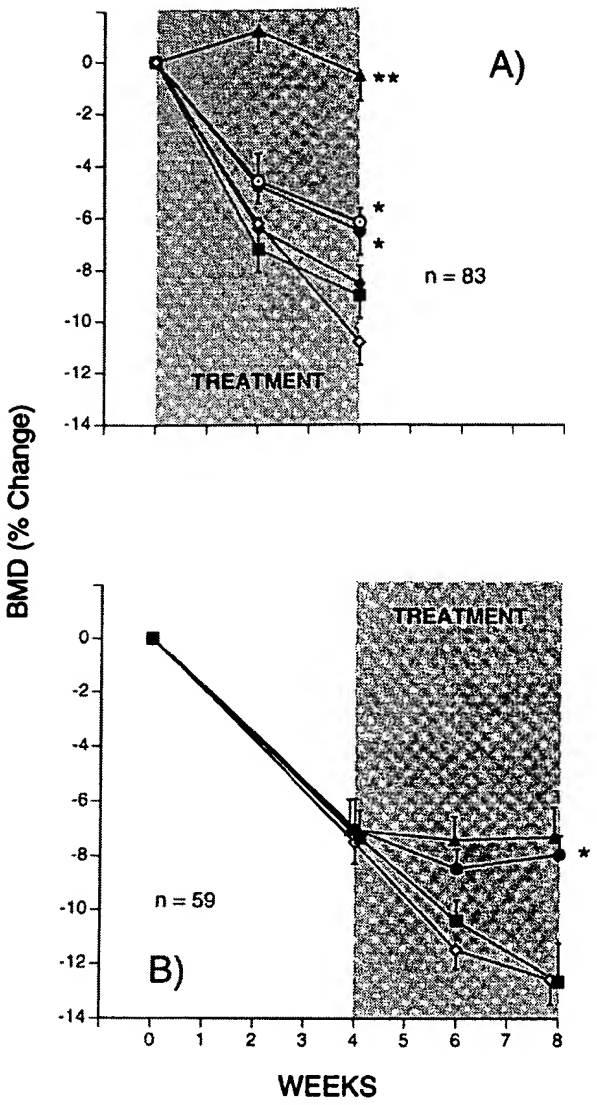

Figure 2. Effect of IL-1ra treatment on distal femur BMD (mean±SEM). Results are expressed as percent change from baseline. In experiment 1 (panel *A*) treatments were started at the time of ovariectomy. In study 2 (panel *B*) treatments were started 4 wk after ovariectomy. Analysis by ANOVA for repeated measures and multiple range tests (Fisher protected LSD test) showed that at the end of each study ovariectomized rats treated with 2 mg/kg body wt per d IL-1ra (●) (*A* and *B*) or 10 mg/kg body wt per d IL-1ra (⊙) (*A*) had a smaller decrease (*$P < 0.05$) in BMD than rats treated with IL-1ra vehicle (■) or BSA (◇), whereas those treated with 0.4 mg/body wt per d IL-1ra (♦) (*A*) had not. At the end of study 1 (*A*) the IL-1ra and estrogen groups (▲) were also significantly different (**$P < 0.05$). In study 2 (*B*) there was no difference between the IL-1ra and the estrogen group at both 2 and 4 wk of treatment. Each treatment group consisted of a similar number of rats. Value *n* represents the total number of rats which completed the corresponding experiment and which were subjected to BMD measurements at each time point. The baseline BMD values (100%) were as follows: experiment 1: vehicle, 298±3.4 mg/cm^2; BSA, 299±3.7 mg/cm^2; 0.4 mg/body wt per d IL-1ra, 294.9±3.4 mg/cm^2; 2 mg/kg body wt per d IL-1ra, 293.1±3.1 mg/cm^2; 10 mg/body wt per d IL-1ra, 298.5±3.4 mg/cm^2; estrogen, 296.6±3.4 mg/cm^2. Experiment 2: vehicle, 300.3±3.0 mg/cm^2; BSA, 299±3.2 mg/cm^2; 2 mg/kg body wt per d IL-1ra, 297.9±2.4 mg/cm^2; estrogen, 296.1±3.4 mg/cm^2.

Table II. Effect of IL-1ra Treatment on Distal Femur BMD (Percent Change from Baseline) in Sham-operated Rats

Group	Study 1: treatment started at surgery (n = 27)			Study 2: treatment started 4 weeks after surgery (n = 30)		
	BMD before surgery	BMD at 2 wk of treatment	BMD at 4 wk of treatment	BMD before surgery	BMD at 2 wk of treatment	BMD at 4 wk of treatment
	mg/cm²	*% change*		*mg/cm²*	*% change*	
BSA	294.9±4.1	−0.7±0.7	−3.8±0.4	301.1±3.7	−2.3±0.9	−2.6±0.8
Vehicle	295.8±4.5	−1.7±1.3	−3.4±0.8	300.2±3.8	−3.6±1.4	−2.4±0.8
IL-1ra	294.4±3.9	−1.2±1.4	−3.4±0.5	297.7±4.4	−3.6±0.5	−3.6±0.5

Sham operation did not cause significant BMD changes in either of the two experiments. There were no significant differences between the vehicle-, and BSA-, and the IL-1ra-treated sham-operated rats. All experimental procedures and statistical analysis were conducted as described in Fig. 2. In study 1 rats were treated with 10 mg/kg body wt per d IL-1ra, 10 mg/kg body wt per d BSA, or IL-1ra vehicle. In study 2, rats were treated with 2 mg/body wt per d IL-1ra, 2 mg/kg body wt per d BSA or IL-1ra vehicle. Values are ±SEM.

During the second month after ovariectomy TBV decreased at a low rate in the vehicle-treated ovariectomized rats. As a result, the difference in TBV between baseline controls (killed at week 4) and the vehicle group (killed at week 8) slightly miss significancy ($P = 0.058$) (Fig. 3 *A*). However, at week 8 the vehicle-treated ovariectomized rats had a lower TBV than both the vehicle and the IL-1ra-treated sham rats. These findings indicate that ovariectomy induced a significant loss of trabecular bone and that a large part of this loss occurred in the first 4 wk after ovariectomy. Estrogen was effective in preventing TBV loss during the treatment period, as indicated by the finding of a similar TBV in the estrogen-treated rats and the baseline control group. However, because of the small TBV loss in the vehicle group between weeks 4 and 8, at the end of the study TBV was not significantly higher in the estrogen group than in the vehicle group. Treatment with IL-1ra was also effective in preventing trabecular bone loss during the second month after ovariectomy, as demonstrated by the finding of a higher TBV in the IL-1ra-treated group than in the baseline control group. Although this difference was not significant, as a result of the increase in TBV induced by IL-1ra between weeks 4 and 8, at the end of the study TBV was significantly higher in the IL-1ra group than in the vehicle-treated rats. Taken all together, these data suggest that IL-1ra was more potent than estrogen in preventing trabecular bone loss between weeks 4 and 8.

In vehicle-treated rats, ovariectomy was also associated with a significant increase in osteoclast number (Fig. 3 *B*) and osteoclast covered surfaces (Fig. 3 *C*). The lack of a difference between the vehicle group and the baseline controls indicate that bone resorption increased mainly during the first 4 wk after ovariectomy. The increase in these two indices of bone resorption was equally prevented by either IL-1ra or estrogen. IL-1ra had no effect on TBV and indices of bone resorption in sham-operated rats.

The effect of IL-1ra on trabecular bone formation was evaluated by measuring static and dynamic (tetracycline-based) indices. Although metaphyseal endocortical surfaces showed an amount of double-labeled surfaces sufficient for precise quantification of dynamic indices of bone formation, < 0.5% of the metaphyseal trabecular surface utilized for the histomorphometric analysis exhibited double-tetracycline labeling, a finding consistent with a low rate of trabecular bone formation. Although the small amount of double-labeled surfaces prevented a precise estimation of the rate of bone formation, the mineralized surface corresponded to the osteoid surface and estrogen, but not IL-1ra or vehicle, appeared to decrease tetracycline-labeled trabecular surface (not shown). Analysis of static indices of bone formation showed that in sham-operated rats the amount of osteoid surface and the osteoid thickness were small (Table III) in both the vehicle and IL-1ra treated rats. These findings are similar to those of Wronski et al. (48) and confirm that in aged rats bone formation takes place at a low rate. Although both indices increased slightly after ovariectomy, the differences were not significant. Moreover, neither IL-1ra nor estrogen treatment induced significant changes in these indices.

Analysis of cortical bone (Table IV) by histomorphometry of tibial diaphysis harvested at week 8 revealed that ovariectomy had no significant effects on cortical bone area, cortical thickness, marrow area, and cross-sectional area. Moreover, although the finding of a small amount of double-labeled surfaces prevented us from accurately measuring endosteal and periosteal bone formation rate, there was sufficient single labeling to accurately measure the percentage of labeled surface, a dynamic index of bone formation. We found that ovariectomy caused a significant increase in endosteal and periosteal labeled surfaces. Estrogen treatment between weeks 4 and 8 had no effect on static indices of bone and marrow areas. However, estrogen treatment induced a significant decrease in periosteal but not endosteal labeled surfaces. In contrast, IL-1ra had no effect on both static and dynamic indices.

Effect of IL-1ra treatment on urinary excretion of pyridinoline crosslinks and blood osteocalcin levels. Bone turnover is known to increase rapidly in response to estrogen withdrawal in both humans and rats (43). Therefore, in order to investigate the effects of IL-1ra on the early changes in bone turnover induced by ovariectomy, the urinary excretion of pyridinoline crosslinks, a marker of bone resorption (39, 40), and the serum levels of osteocalcin, a marker of bone formation (43), were measured 2 and 4 wk after ovariectomy. At 2 wk after surgery, pyridinoline crosslink excretion and serum osteocalcin were both significantly higher in ovariectomized rats treated with vehicle than in sham-operated rats (Figs. 4 and 5). At 4 wk both indices were higher ($P < 0.05$) than at 2 wk, indicating that bone turnover continued to increase during the first

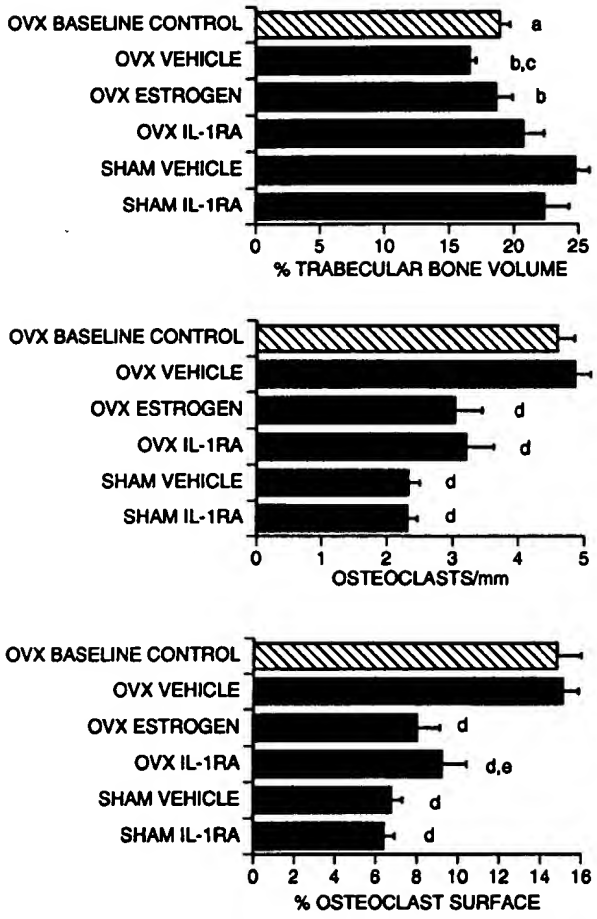

Figure 3. Effect of IL-1ra treatment (mean±SEM) on TBV and histomorphometric indices of bone resorption. Rats treated with either vehicle, IL-1ra or estrogen (n = 10 rats per group, randomly selected from the entire experimental group) between weeks 4 and 8 (study 2) were killed at the end of week 8 (*solid bars*) and compared to untreated rats (n = 6) killed 4 wk after ovariectomy (baseline controls; *hatched bars*). Distal femurs were harvested and processed as described in the methods. Percent trabecular bone volume is the percentage of marrow space occupied by trabecular bone. Osteoclasts are given as the number of osteoclasts per millimeter of trabecular bone surface. Percent osteoclast surface is the percentage of trabecular surface containing osteoclasts with "resorptive bays." [a] $P < 0.05$ compared to sham vehicle; [b] $P < 0.05$ compared to sham vehicle and sham IL-1ra; [c] $P < 0.05$ compared to ovx IL-1ra; [d] $P < 0.05$ compared to ovx baseline and ovx vehicle; [e] $P < 0.05$ compared to sham IL-1ra by Fisher protected LSD test.

month after ovariectomy. Treatment with IL-1ra started at the time of surgery decreased pyridinoline excretion in ovariectomized rats (Fig. 4). At both 2 and 4 wk after ovariectomy pyridinoline excretion was, in fact, lower in rats treated with either IL-1ra or estrogen than in those treated with vehicle. At 2 wk there was no difference between the IL-1ra and the estrogen-treated rats. At 4 wk, a time point when bone resorption was higher, pyridinoline excretion was lower in the estrogen than in the IL-1ra-treated ovariectomized rats. In sham-operated rats IL-1ra treatment had no significant effects on pyridinoline excretion at both 2 and 4 wk.

The increase in serum osteocalcin induced by ovariectomy was prevented by estrogen treatment, but not by IL-1ra treatment. In ovariectomized rats at both 2 and 4 wk osteocalcin levels were, in fact, significantly lower in the estrogen-treated rats than in either the IL-1ra or the vehicle group. In addition, IL-1ra treatment had no effect on osteocalcin levels in sham-operated rats.

Discussion

In this study we have found that treatment of ovariectomized rats with IL-1ra decreases bone resorption and blocks ovariectomy-induced bone loss.

At the doses used in this study IL-1ra competes specifically with IL-1 and does not possess IL-1 agonistic effects (49). In fact, infusions of IL-1ra up to 80 mg/kg body wt per d to healthy humans have been shown to cause no detectable biological effects (50). The subcutaneous infusion of IL-1ra resulted in serum IL-1ra levels ~ 10 times higher than those required to block ^{45}Ca release in vitro from rat bone stimulated with 10 ng/ml of IL-1 (33). Although there is no published information on either the amount of IL-1 released in vivo after ovariectomy or the level of IL-1 receptors expressed in rat bone cells, the need for such a high concentration of IL-1ra to affect bone loss in vivo is not surprising, as it is known that biological responses to IL-1 can be observed when ≤ 5% of IL-1 receptors are occupied by IL-1 (30, 49). Interestingly, the anti-IL-1 activity of sera from rats treated with IL-1ra for 4 wk was similar to that of fresh IL-1ra. This indicates that the 4-wk-long infusion did not result in the production of rat anti-human IL-1ra antibodies in amounts sufficient to block the biological activity of IL-1ra or that IL-1ra treatment induced the formation of non-blocking antibodies.

The effects of IL-1ra on TBV and bone density were evaluated by bone histomorphometry and by dual-energy X-ray absorptiometry, a noninvasive technique which provides precise integrated measures of cortical and trabecular bone (35). These measures were carried out in the distal femur, a weight bearing skeleton segment rich in trabecular bone. In agreement

Table III. Effect of IL-1ra on Histomorphometric Indices of Trabecular Bone Formation

Parameter	Sham vehicle (n = 10)	Sham IL-1ra (n = 10)	Ovx vehicle (n = 10)	Ovx IL-1ra (n = 10)	Ovx estrogen (n = 10)
Percent osteoid surface*	0.56±0.19	0.64±0.23	1.16±0.39	0.41±0.23	0.52±0.20
Osteoid thickness‡ (μm)	1.15±0.33	1.07±0.32	1.33±0.31	1.35±0.39	1.22±0.33

Mean±SEM. * Percentage of trabecular surface covered by osteoid. ‡ Average osteoid width.

Table IV. Effect of IL-1ra on Cortical Bone

Group	Marrow area	Cross-sectional area	Cortical bone area	Cortical thickness	Endosteal labeled surface	Periosteal labeled surface
	mm^2	mm^2	mm^2	mm	%	%
Sham IL-1ra	2.45±0.18	7.13±0.24	4.67±0.09	0.52±0.01	0.60±0.37	0.42±0.12
Sham vehicle	2.56±0.09	7.23±0.17	4.66±0.10	0.51±0.01	1.09±0.44	0.74±0.24
Ovx vehicle	2.50±0.15	7.21±0.23	4.66±0.15	0.51±0.01	4.50±1.22[a]	15.8±1.99[b]
Ovx E2	2.18±0.14	6.83±0.37	4.65±0.24	0.53±0.02	2.55±0.83	1.69±0.73
Ovx IL-1ra	2.57±0.20	7.25±0.29	4.67±0.10	0.51±0.01	5.57±0.69[a]	14.4±2.84[b]

Values are mean±SEM. n = 6 rats per group. [a] $P < 0.05$ compared to sham vehicle and sham IL-1ra. [b] $P < 0.05$ compared to sham vehicle, sham IL-1ra, and ovariectomized (Ovx) estrogen (E2).

with earlier studies (51–53), both methods showed that ovariectomy causes a marked bone loss. However, because of the lower variability, a shorter follow-up was required to detect a significant bone loss with dual-energy X-ray absorptiometry than with bone histomorphometry. Our findings are consistent with those of earlier studies demonstrating both the higher sensitivity for bone mass quantification and the higher correlation with density measurements by physical means, of X-ray absorptiometry than bone histomorphometry (54, 55).

The bone-sparing effect of IL-1ra was more potent in the second than in the first month after ovariectomy, a time when bone marrow cell production of IL-1 bioactivity was the highest. In fact, bone loss was decreased, but not completely arrested, when rats were treated with IL-1ra in the early postovariectomy period. In contrast, bone loss was completely arrested when rats were treated with IL-1ra during the second month after ovariectomy. These data suggest that the role of IL-1 in conditioning the changes in bone remodeling induced by estrogen deficiency increases with the passage of time since ovariectomy. This hypothesis is further supported by the finding that in each study IL-1ra was also more effective during the second 2 wk than in the first 2 wk of treatment. In study 1, the bone sparing effects of IL-1ra at 2 or 10 mg/kg body wk per d were similar. An insufficient block of IL-1 activity is, therefore,

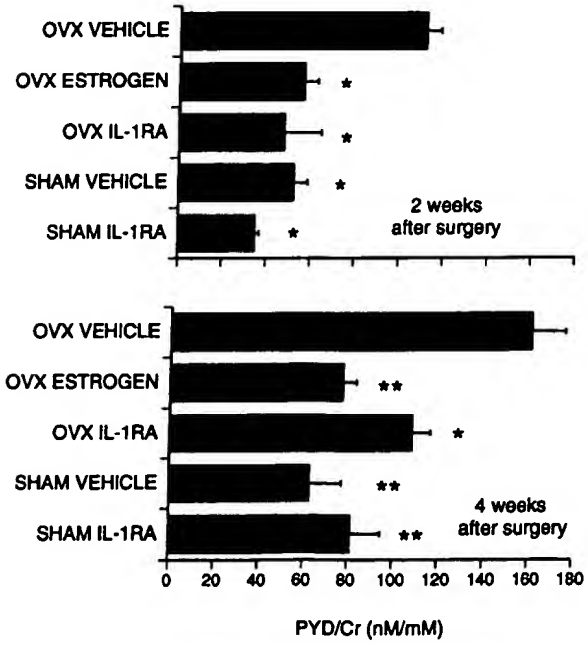

Figure 4. Effect of IL-1ra treatment on the urinary excretion (mean±SEM) of pyridinoline crosslinks (n = 8 rats per group, randomly selected from the entire experimental group). The pyridinoline crosslinks/creatinine ratio (PYD/Cr) was determined in urine samples collected 2 and 4 wk after surgery as described in Methods. Rats were treated with IL-1ra during the first 4 wk after surgery. *$P < 0.05$ compared to vehicle treated ovariectomized rats. **$P < 0.05$ compared to both vehicle- and IL-1ra-treated ovariectomized rats, by Fisher protected LSD test.

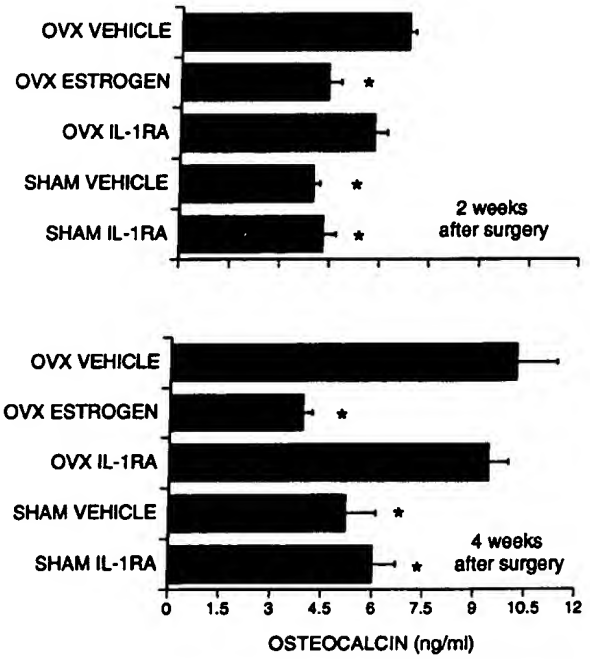

Figure 5. Effect of IL-1ra treatment on the serum levels (mean±SEM) of osteocalcin (n = 8 rats per group, randomly selected from the entire experimental group). Osteocalcin was measured in serum samples collected 2 and 4 wk after surgery from rats treated with IL-1ra during the first 4 weeks after surgery. *$P < 0.05$ compared to both vehicle- and IL-1ra-treated ovariectomized rats by Fisher protected LSD test.

an unlikely explanation for the inability of IL-1ra to completely block bone loss in the early postovariectomy period.

To investigate the mechanism of the bone-sparing effect of IL-1ra we have examined histomorphometric and biochemical indices of bone turnover. During the second month after ovariectomy IL-1ra treatment resulted in a slight increase in TBV. In contrast, estrogen blocked, but did not reverse, trabecular bone loss. Interestingly, neither IL-1ra nor estrogen affected cortical bone area. These data suggest that at week 8 the IL-1ra- and the estrogen-treated rats had similar BMD values because the difference in trabecular bone density was too small to be detected by our densitometric technique, a method which is heavily influenced by cortical bone and provides an integrated measure of the density of the two skeleton envelopes.

Although we were unable to accurately quantitate trabecular bone formation, analysis of cortical bone by bone histomorphometry and of serum osteocalcin levels indicated that estrogen, but not IL-1ra, prevented the increase in indices of bone formation which typically follows ovariectomy in rats (37, 56). We recognize that the different effects of the two agents on bone formation should be interpreted with caution. However, one may speculate that estrogen modulates bone resorption via an IL-1–dependent pathway and bone formation via a distinct, IL-1–independent, mechanism.

Our findings also demonstrate that IL-1ra decreases bone resorption in a manner similar to estrogen replacement. Interestingly, the effect of IL-1ra on pyridinoline crosslink excretion during the first month after ovariectomy was more potent than that observed in the same time period with BMD measurements. This is consistent with previous human studies demonstrating that biochemical markers of bone turnover do not faithfully reflect the rate of bone loss at the time of the marker measurement, but rather correlate moderately with BMD measurements (43).

IL-1ra had no effect on the bone density and on the bone turnover of sham-operated rats, indicating that IL-1ra specifically blocks estrogen-dependent bone loss. The data are also consistent with the lack of published evidence for a role of IL-1 in physiologic bone remodeling.

Taken all together, our findings demonstrate that IL-1 contributes to the pathogenesis of postovariectomy bone loss. However, the incomplete response to IL-1ra observed in the early postovariectomy period suggests that other factors may contribute to the bone loss induced by estrogen withdrawal. A likely candidate is IL-6. This cytokine is, at least in the mouse, regulated by estrogen (26), and an increased production of IL-6 in the bone marrow of ovariectomized mice increases osteoclastogenesis in vitro (18). Thus, since the secretion of IL-6 from stromal cells and bone cells is induced by both IL-1 and TNFα (26), our findings could, indeed, be explained by a persistent production of IL-6. Conversely, the similar response to estrogen and IL-1ra in the second month after ovariectomy, suggests that the production of, or the response to, IL-6 or other cytokines produced independently of IL-1 may decrease in the late postovariectomy period.

In conclusion, the present findings indicate that IL-1, or mediators induced by IL-1, play an important causal role in the mechanism by which ovariectomy induces bone loss in rats, especially following the early postovariectomy period. It will be interesting, therefore, to investigate whether IL-1ra has a practical role in the treatment of postmenopausal osteoporosis in humans.

Acknowledgments

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PTH → BF ↑
BR no change

Temporal Expression of the Anabolic Action of PTH in Cancellous Bone of Ovariectomized Rats

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ABSTRACT

When administered intermittently, parathyroid hormone (PTH) is a potent anabolic agent in both human and animal bone. To improve our understanding of this anabolic effect, we have examined the time course of PTH action in an established animal model of estrogen deficiency-induced bone loss: the ovariectomized rat. Animals were ovariectomized (Ovx) and allowed to lose bone for 6 weeks. A dose of 20 μg/kg/d of rat PTH (1-34) was administered s.c., 6 days each week for periods of 1, 2, 3, 4, 6 and 8 weeks. Animals were sacrificed for evaluation of skeletal histomorphometry of the proximal tibia and mechanical strength of the cancellous bone in the marrow cavity of the distal femur. Cancellous bone volume (Cn-BV/TV) increased gradually over 8 weeks of treatment (16.8 ± 1.6 to 24.1 ± 2.7%) as did the bone formation rate (0.308 ± 0.054 to 1.659 ± 0.293 $\mu m^3/\mu m^2/d$), as determined by an increase in both total mineralization surface (15.5 ± 2.1 to 42.7 ± 5.0%) and mineral apposition rate (1.88 ± 0.20 to 3.55 ± 0.39 μm/d). The largest increments in these variables reflecting bone formation occurred over the first week of treatment. This bone formation was accompanied by an increase in trabecular thickness (Tb.Th) (55.3 ± 3.4 to 80.5 ± 5.0 μm) without a corresponding increment in trabecular number (Tb.N) (3.65 ± 0.17 to 3.55 ± 0.26). Extensive tetracycline labels were visualized on the surface of trabecular rod-like and plate-like structures. A small transient, though not statistically significant, increase occurred in both eroded surface and urinary pyridinoline concentration immediately after the onset of PTH administration. Osteocalcin showed a small decrement in the first two weeks after PTH administration, but the levels were elevated when compared with the Ovx control in later weeks. Mechanical strength of the cancellous bone also increased significantly with PTH treatment (20.5 ± 2.4 to 46.1 ± 10.0 Newtons). Our results showed that: 1) intermittent PTH treatment of Ovx rats elicited an immediate increase of bone formation activity by the existing osteoblasts, 2) the increase of Cn-BV/TV after PTH administration resulted primarily from an increase in Tb.Th, and 3) improved mechanical strength after PTH treatment can be achieved by increases in Tb.Th without an increase in Tb.N. (J Bone Miner Res 1996;11:421–429)

INTRODUCTION

POST-MENOPAUSAL OSTEOPOROSIS is a world-wide problem of staggering proportions. Two basic means of treatment have been used to combat bone loss, namely, inhibition of bone resorption and stimulation of bone formation.[1] Although anti-resorptive agents such as estrogen can transiently increase bone mass and prevent further bone loss,[2] an anabolic agent that promotes bone gain would be an invaluable addition to therapeutic approaches to patients with osteoporosis.

In humans, fluoride[3–5] and intermittent PTH[6–9] administration have been tested and apparently are capable of improving vertebral bone mass. Clinical studies with fluoride show an increase in vertebral bone mass, but results from fracture studies have been inconsistent. Human PTH

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(1-34), in both uncontrolled trials and one controlled study, also appears to increase vertebral bone mass in osteoporotic subjects, but no fracture data have been published. The recent findings of improved mechanical competency as a result of PTH treatment in rat vertebrae,[10] femoral neck bones,[11] and appendicular cortical bones[12] makes the use of PTH a potentially attractive choice for the treatment of established osteoporosis.

The ovariectomized rat recently has received substantial acceptance as a surrogate for the study of human postmenopausal osteoporosis.[13,14] The model is best suited for studying cancellous bone changes during drug treatment and we and others have recently reviewed the anabolic effects of PTH in this model.[15,16] In general, PTH can increase cancellous and cortical bone mass by stimulating bone formation without the need for an accompanied elevation in bone resorption.[17,18] Several studies[11,19-21] have examined PTH action on bone mass and/or mechanical strength at two or three different time points, but little is known about the relationship between the improvement of mechanical strength and the changes in bone structure. In a recent study, Li et al.[22] reported that compressive strength correlated with bone mass and trabecular thickness in lumbar vertebrae of PTH treated rats. In this study, we examined the effects of PTH treatment in a temporal fashion, with special emphasis on the relationships among bone mass, structure, and mechanical strength.

MATERIALS AND METHODS

Animals

Eighty four-month-old virgin female Sprague-Dawley rats were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN, U.S.A.). The rats were maintained at the Animal Research Facility at Helen Hayes Hospital for two months to allow them to adapt to their new environment. Animals were fed Purina Laboratory Rodent Chow containing 1% Ca (Purina Mills, St. Louis, MO, U.S.A.) and tap water ad libitum throughout the experiment.

Experimental protocol

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Helen Hayes Hospital. Eighty rats were randomly subdivided into two groups of sham-operated and eight groups of bilaterally ovariectomized rats, with 8 animals in each group. Animals were anesthetized with 12.5 mg Ketamine (Ketalar, Parke-Davis, Morris Plains, NJ, U.S.A.) and 2.5 mg xylazine hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.), administered i.p.. After the operation, six weeks were allowed to pass before initiation of treatment in order to permit significant bone loss to occur in the Ovx groups. Six Ovx groups were then given s.c. injections of 20 μg/kg/d of rat PTH (1-34) (Bachem Inc., Torrance, CA, U.S.A.), 6 days a week. PTH-treated animals were sacrificed 1, 2, 3, 4, 6, and 8 weeks after the onset of treatment. Ovx and sham control groups were given vehicle only (1 mM glacial acetic acid) and were sacrificed only at the beginning and the end of the treatment periods. Two doses of calcein (10 mg/kg, Sigma Chemical Co.) were injected i.p. into each animal 6 and 2 days prior to sacrifice in order to visualize new bone formation. A 24 h urine sample was collected via metabolic cages the day before sacrifice. Each animal was anesthetized with ketamine: xylazine anesthetic and sacrificed by exsanguination via the abdominal aorta. Blood, right tibia, and right femur were obtained from each animal.

Measurement techniques

Osteocalcin and pyridinoline assays: Rat serum osteocalcin concentrations were measured with a commercial radioimmunoassay kit (Biomedical Technologies Inc., Stoughton, MA, U.S.A.) using rat osteocalcin as standard and a region-specific domain of goat anti-rat osteocalcin antibody. Urinary free pyridinoline and creatinine were measured by an ELISA kit (Pyrilinks, Metra Biosystems, Mountain View, CA, U.S.A.). Intra-assay coefficients of variation for rat osteocalcin and pyridinoline were 6 and 12%, respectively.

Histomorphometric analysis: Excised right tibiae were dehydrated, embedded undecalcified in methyl methacrylate, and sectioned longitudinally using a Jung Ultracut microtome (Reichert-Jung, Heidelberg, Germany). Longitudinal sections from the center of each bone measuring 10 μm were obtained and mounted unstained or were stained by the Goldner's trichrome method. Two consecutive sections, one unstained and one stained, were measured by a direct tracing method using an interactive measuring system (Osteomeasure, Osteometrics, Atlanta, GA, U.S.A.). Static and dynamic histomorphometric parameters were obtained from a 2 × 2 mm field positioned 1 mm distal to the lowest point of the growth plate in the metaphyseal region. Static parameters were measured with a 4× objective, whereas dynamic parameters were measured with a 20× objective. Cancellous bone volume (Cn-BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), mineralizing surface (MS/BS = 1/2 SLS/BS + DLS/BS), mineral apposition rate (MAR) and bone formation rate (BFR = MAR * MS/BS) were calculated by the Osteomeasure software, according to the convention of standardized nomenclature.[23] The number of double labeled sites and the average length of double labeled sites were also calculated by the Osteomeasure software.

To visualize the microarchitecture of the newly mineralized bone, 30 serial sections of the proximal tibia in selected PTH treatment groups were obtained. Cancellous bone micro-architecture was examined using a series of images captured by imaging software (Optimas, BioScan Inc., Edmonds, WA, U.S.A.). To observe the location of the bone sites undergoing bone mineralization, photomicrographs of double labeled surfaces were taken under fluorescent light. The remaining tibiae were deplasticized, coated with gold, and examined by scanning electron microscopy as described previously.[24]

Mechanical testing of the femur: Excised femurs were subjected to an indentation test using a Materials Testing System (Model 810, MTS Systems Corp., Minneapolis, MN, U.S.A.), employing a modification of the method used to

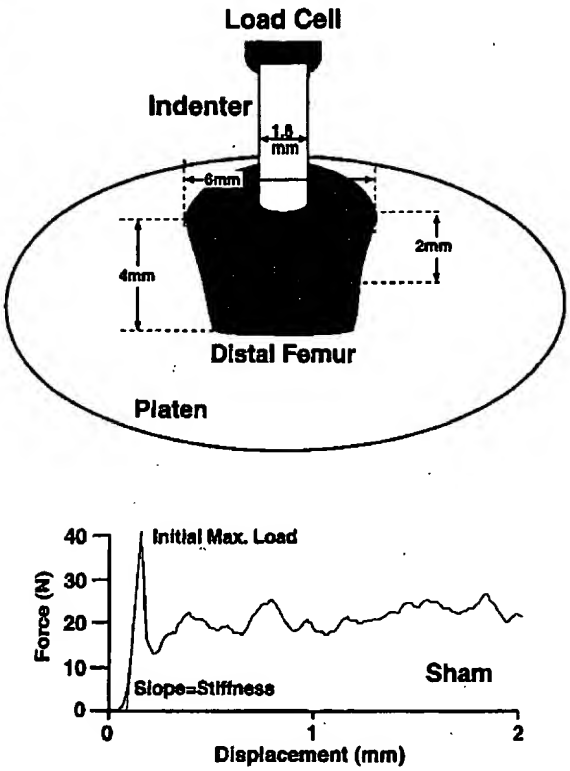

FIG. 1. Representation of the load cell and indenter positions and dimensions employed in the indentation test to assess load and stiffness in the cancellous bone of the distal femur. The graph displays a representative load-displacement curve in a Sham-operated animal. Details of the procedure are given in the text.

determine mechanical strength of knee replacements in humans.[25,26] A 4 mm section of the distal femur was cut directly proximal to the femoral condyle with a low speed saw (Isomet, Buehler LTD, Lake Bluff, IL, U.S.A.) under constant saline irrigation. A cylindrical indenter with a flat testing face 1.6 mm in diameter, equivalent to approximately less than 50% of the diameter of the marrow cavity, attached to a 25 lb load cell (MTS Model 3397-101), was applied to the center of the marrow cavity on the distal face of the section at a constant displacement velocity of 0.1 mm/s. The indenter was allowed to penetrate the cavity to a depth of 2 mm before load reversal (Fig. 1). During placement and testing, normal saline was regularly applied to the specimens to prevent drying. Load-deformation values were recorded on disk, and the initial maximum load and stiffness were calculated from the magnitude of the first load peak and the slope of the initial load curve.

Statistical analysis

The effects of treatment, treatment periods, or aging were analyzed by ANOVA, using Duncan's multiple group comparison procedure, with SAS software (SAS Institute Inc., Cary, NC, U.S.A.). A p value of 0.05 was used to determine statistical significance.

RESULTS

Histomorphometry and biochemical markers

In general, sham-operated, vehicle treated animals showed little change in any of the structural variables of cancellous bone (Fig. 2). Cn-BV/TV of Ovx animals decreased significantly after 6 weeks (18.6 ± 1.3% in Ovx vs 32.6 ± 2.2% in Sham) and further decreased when treated with vehicle for 8 weeks (4.7 ± 0.8% in Ovx vs. 28.8 ± 1.1% in Sham) (Fig. 2). This change in Cn-BV/TV was accompanied by a decrease in Tb.N and a substantial increase in Tb.Sp, with little change in Tb.Th. The lines connecting the two Sham and Ovx groups in each of the graphs are dotted to denote the uncertainty of the intermediate values.

PTH treatment of Ovx rats for 8 weeks, which began 6 weeks after the operation, resulted in a time-dependent increase in Cn-BV/TV beginning as early as the third week of treatment. Increases in Cn-BV/TV (16.8 ± 1.6 to 24.1 ± 2.7%) resulted from an increase in Tb.Th (55.3 ± 3.4 to 80.5 ± 5.0 μm) rather than a change in Tb.N (3.65 ± 0.17 to 3.55 ± 0.26) or Tb.Sp (228.3 ± 17.5 to 223.5 ± 29.8 μm) at all time points examined (Fig. 2). MS/BS and MAR were both greater in Ovx rats treated with PTH and resulted in an elevated BFR (0.971 ± 0.090 to 1.758 ± 0.239 $\mu m^3/\mu m^2/d$ (Fig. 3). The increase in MS/BS resulted from an increase in both the number of sites as well as the average length of such sites (data not shown). The bone formation marker osteocalcin decreased in the first two weeks of treatment, then returned to a level higher than the Ovx control group after the second week (Fig. 4). Eroded surface showed a trend toward an increase, although this trend was not statistically significant, in the first 3 weeks after the initiation of PTH treatment. The early increases were accompanied by a small, not significant increase in the collagen breakdown product, pyridinoline (Figs. 3 and 4). The levels subsequently returned to Sham (PYD) or reduced levels (ES/BS) thereafter.

Mechanical competency

The initial maximum load, i.e., the load at which initial deformation of cancellous bone occurs, increased within the first three weeks of PTH treatment (Fig. 5) and continued to increase steadily up to week 6 of the treatment period. The stiffness of the bones displayed a similar pattern. Both the load and stiffness in Ovx animals treated with vehicle were significantly lower than in the Sham or long term PTH-treated animals (load: 5.27 ± 0.70 N in Ovx, 34.7 ± 2.0 N in Sham and 46.1 ± 10.0 N in Ovx + PTH; stiffness: 119.3 ± 14.2 N/mm in Ovx, 534.8 ± 7.0 N/mm in Sham and 531.6 ± 107.0 N/mm in Ovx + PTH (Fig. 5). A significant correlation was found between cancellous bone mass, trabecular thickness, and initial maximal load (Fig. 5). There seemed to be a slowing of PTH action evidenced by a plateau in Cn-BV/TV, mechanical load and stiffness, and

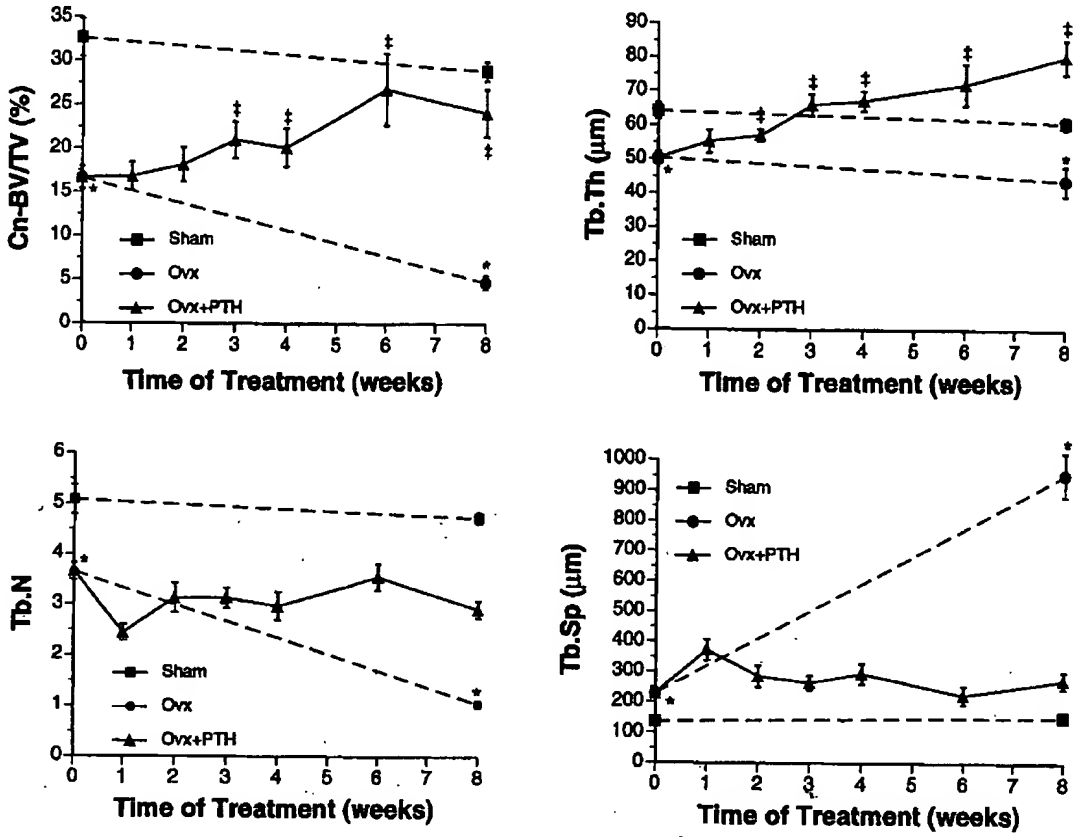

FIG. 2. Effects of intermittent administration of rPTH (1-34) on Cn-BV/TV, Tb.Th, Tb.N and Tb.Sp. Abbreviations are given in the text. Results are reported as mean ± SEM. Statistically significant differences at $p < 0.05$ are indicated by * for Sham vs. Ovx and ‡ for Ovx baseline vs. Ovx + PTH.

decreased tetracycline labeling at week 8 when compared with week 6 (results not shown).

Visualization of the sites of new bone formation

Photographs of fluorescence emitted by the calcein labels were taken to visualize the area of new bone formation. Many double labeled surfaces were observed around the edge of rod-like structures and on the surface of trabecular plates (Fig. 6). In addition, new bone formation was seen at the edge of small fenestrations in the trabecular plates. Photographs of fluorescent labels from a set of serial sections, taken immediately before the sample was processed for scanning electron microscopy, are shown in Fig. 7 to demonstrate such features.

DISCUSSION

Current FDA-approved modes of treating osteoporosis (estrogen and calcitonin) principally prevent further bone loss with no significant restoration of previously lost bone. For this reason, the anabolic action of intermittent PTH administration on bone has generated a great deal of interest. Although there is a large body of information on the anabolic action of PTH, little is known about the temporal response to PTH early in the treatment phase and how the changes in bone structure may affect its mechanical competency. This study was designed to address these two issues.

Our results confirm previous findings that intermittent administration of PTH (1-34) to Ovx rats results in a significant increase in Cn-BV/TV. Prior to the increase of Cn-BV/TV at 3 weeks, MS/BS and MAR increased immediately after PTH administration. As fluorescent labels were given 2 and 6 days prior to sacrifice, we can deduce that the increase occurred immediately after the first day of PTH administration. The length of each double labeled surface increased to its maximum extent at 1 week, as did the MAR, whereas the number of double labeled surfaces did not reach maximum until the second week. This suggests that PTH can elicit an immediate response from the existing osteoblasts by increasing the MAR, although a delay may exist in recruiting new osteoblasts and initiating new bone formation sites. There was a highly significant increase in the BFR at an early stage of treatment, but Cn-BV/TV did not increase correspondingly. This may be partly the result of a temporary increase in bone resorption activity,

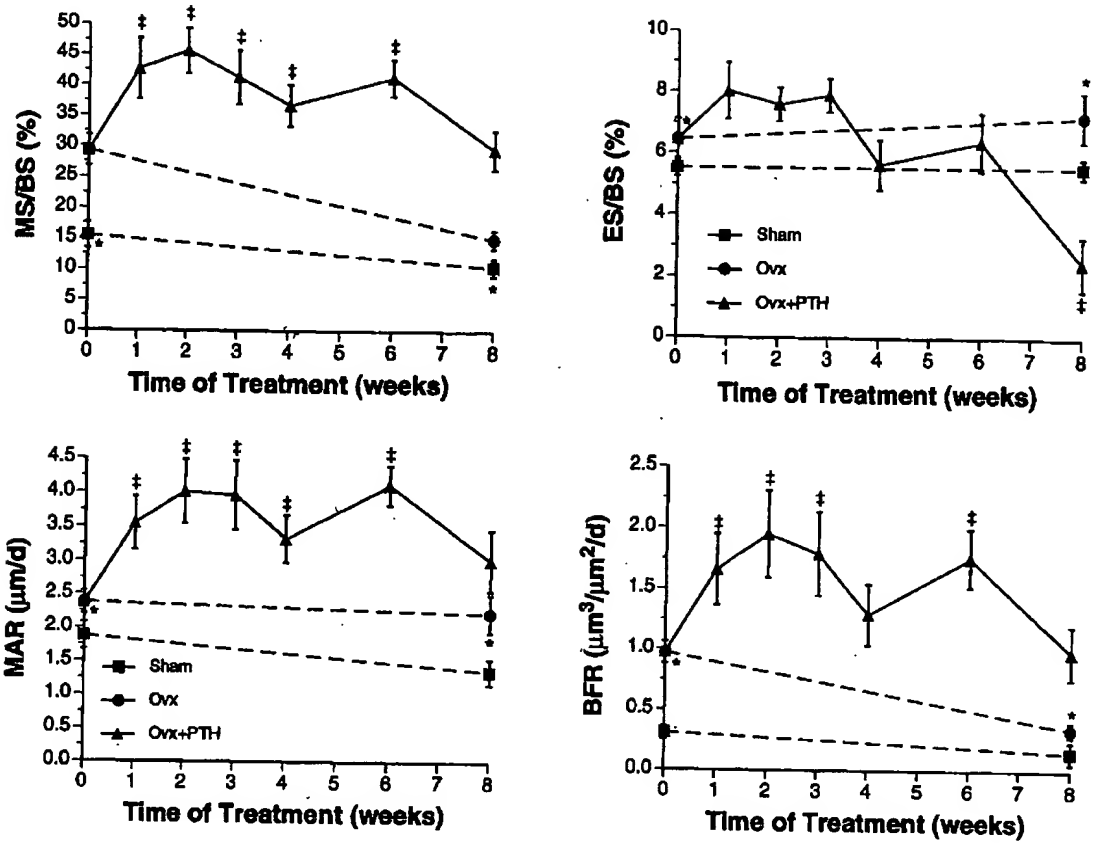

FIG. 3. Effects of intermittent administration of rPTH (1-34) on MS/BS, MAR and BFR. Abbreviations are given in the text. Results are reported as mean ± SEM. Statistically significant differences at $p < 0.05$ are indicated by * for Sham vs. Ovx and ‡ for Ovx baseline vs. Ovx + PTH.

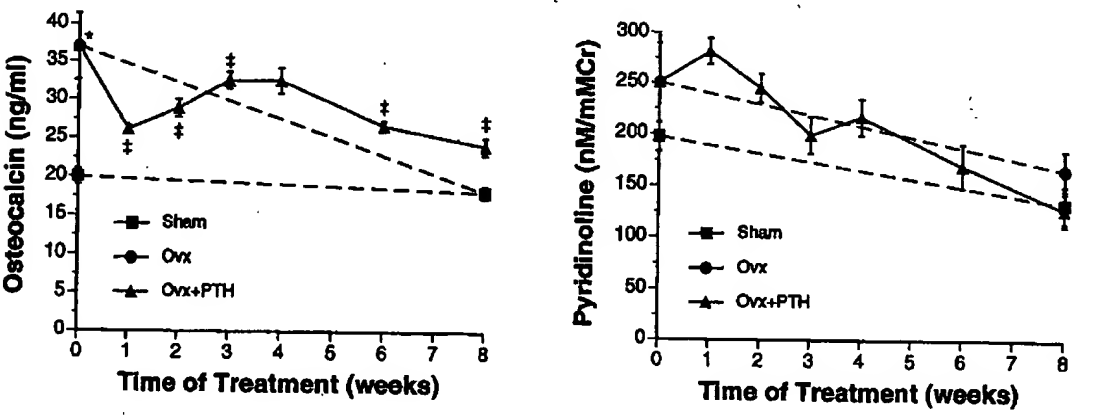

FIG. 4. Effects of intermittent administration of rPTH (1-34) on bone turnover markers, free pyridinoline and BGP. Abbreviations are given in the text. Results are reported as mean ± SEM. Statistically significant differences at $p < 0.05$ are indicated by * for Sham vs. Ovx and ‡ for Ovx baseline vs. Ovx + PTH.

evidenced by an increase in eroded surface and pyridinoline secretion. However, it should be noted that the increments in these variables reflecting bone resorption were not statistically significant. After the third week, Cn-BV/TV and the mechanical strength of the cancellous bone in the marrow cavity began to increase steadily for the next 5 weeks under the influence of continued increases in the BFR and diminished osteoclast action. The plateau of Cn-BV/TV

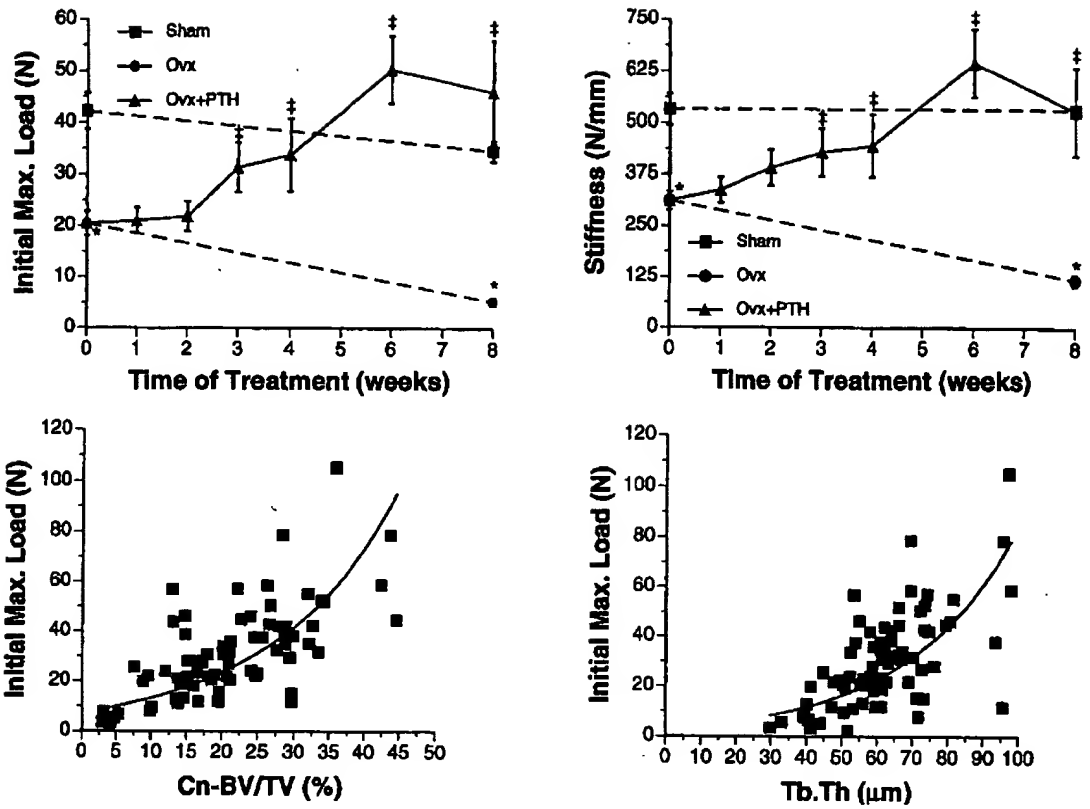

FIG. 5. Effects of intermittent administration of rPTH (1-34) on mechanical competency. Top: Temporal changes of mechanical strength with PTH treatment. Abbreviations are given in the text. Results are reported as mean ± SEM. Statistically significant differences at $p < 0.05$ are indicated by * for Sham vs. Ovx and ‡ for Ovx baseline vs. Ovx + PTH. Bottom: The correlation of mechanical strength with either Cn-BV/TV or Tb.Th.

and mechanical load and stiffness, and the decreases of labeling parameters at week 8 compared to week 6, could signal a slowing or cessation of PTH action. These very limited data suggest that PTH anabolic action may have a limited functional period, at least in one treatment cycle, as described by others in humans[27] and in rats.[10,12]

Our findings of the changes in biochemical markers are very similar to our ongoing clinical study of intermittent PTH administration in humans.[28] In both studies osteocalcin levels underwent an immediate decrease followed by an increase, while the bone resorption marker, pyridinoline, showed an immediate increase followed by a decrease at the later time points. The changes in pyridinoline agree with the observation of an early transient increase in eroded surface during treatment, although the changes were not statistically significant for either variable. The osteocalcin data are more difficult to interpret. The initial decrease after PTH administration could result from the diminished secretion of osteocalcin from individual osteoblasts. The later increase of osteocalcin could result from the increased recruitment of new osteoblasts under the influence of PTH. This suggests that osteocalcin data need to be carefully interpreted during a transient stage of altered bone formation.

New bone formation during PTH treatment could either occur de novo or by apposition onto existing plates. Although we cannot state with certainty which of these two mechanisms is in operation, the following evidence strongly suggests the latter: First, we only observed an increase in Tb.Th, and not in Tb.N, at any time point and second, we did not observe any diffused tetracycline labels, a characteristic of de novo bone formation, at any time point examined. One should note, however, that de novo synthesis of small quantities of woven bone in the marrow cavity was observed following administration of 400 to 1000 μg/kg/d of PTH, a dose that is 20 to 50 times greater than that used here.[20] The question of whether the excess bone formation is initiated randomly on resting bone surfaces, or overfills newly created resorption cavities, is less certain. We did observe an initial increase in eroded surface, but this increase disappeared after 3 weeks, while the BFR remained highly elevated for 8 weeks. A separate analysis using a special labeling strategy, similar to that employed by Tobias et al.,[29] may be needed to address this issue.

A true test of a successful treatment for osteoporosis would be a decrease in fracture incidence. As such a result is difficult to accomplish in the early stages of efficacy

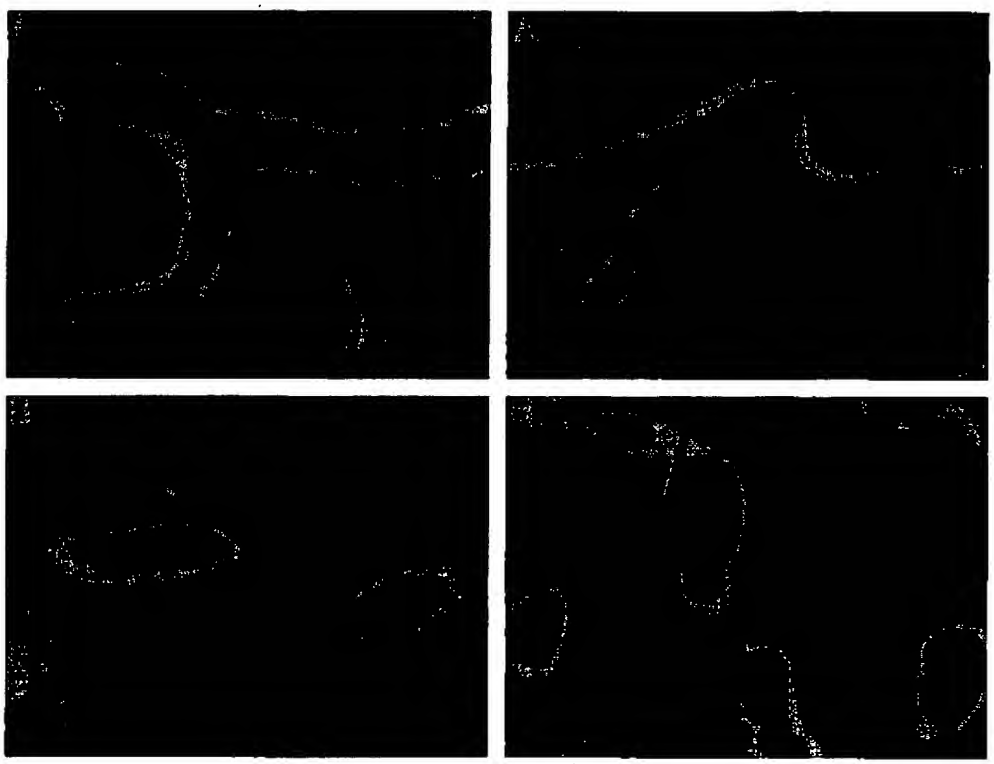

FIG. 6. A visualization of new bone formation in animals treated with intermittent PTH. Fluorescence image of calcein labeled bones from an Ovx animal treated with PTH for 3 weeks. A demonstration of new bone formation on plate-like (A) and rod-like (B) trabecular structures are presented.

testing for new therapeutic agents, mechanical competency testing has been used as a surrogate for this purpose. Mechanical competency depends upon at least two structural determinants of Cn-BV/TV: Tb.Th and Tb.N. Postmenopausal osteoporosis predominantly shows a loss of Tb.N rather than Tb.Th in the iliac crest,[30] whereas bone loss related to the aging process is associated with decrements in Tb.Th.[31] While it is difficult to increase Tb.N, we know that it is feasible to increase Tb.Th with anabolic agents such as fluoride and PTH. It is therefore important for us to examine whether increased Tb.Th, without an improvement in Tb.N, can improve mechanical competency. There are several reports of increased mechanical competency in the cancellous-rich bones of rats after PTH treatment.[10,11,32] One report showed a correlation between compressive strength to bone mass and structure in lumbar vertebrae.[22] However, the data were not analyzed with respect to the changes in structure. From our histomorphometric measurements, we observed that the increase of Cn-BV/TV from PTH treatment was the result of increased Tb.Th rather than an increase in Tb.N. In combination with the observation of a high correlation between mechanical competency and Cn-BV/TV, we suggest that increasing Tb.Th could serve to increase the mechanical strength of the affected cancellous bone without the need for an increase in Tb.N, and presumably, trabecular connectivity.

Our observations on the location of double-labeled surfaces confirm our findings that most of the added bone mass was the result of a thickening of the trabecular plates. We observed many examples of double labels that covered entire trabecular plate surfaces, which would explain the increase in Tb.Th. Double labels, however, were not limited to such surfaces. Examining serial sections of the proximal tibia, we noticed that double labels were also present on the inner surface of small fenestrations of trabecular plates and rod-like structures. These small fenestrations are quite often present in the middle of trabecular plates and are generally in the order of 50 to 200 μm in diameter. Assuming a MAR of 2 to 5 μm per day, as we have found in these animals, it is conceivable that these micro-fenestrations could be closed within 10 to 100 days of PTH administration. Although these microfenestrations are small, and their repaired area represents only a minor fraction of the total bone surface, we should not rule out their possible contribution to the overall improved mechanical competance. The significance of this observation is not fully understood at this time. One possibility is that these structures may represent a weak point in the trabecular plate that is selectively targeted by osteoclasts. Filling such micro-fenestrations may reduce osteoclast action in the future.

In summary, a close monitoring of the early stages of anabolic action of intermittent PTH administration showed

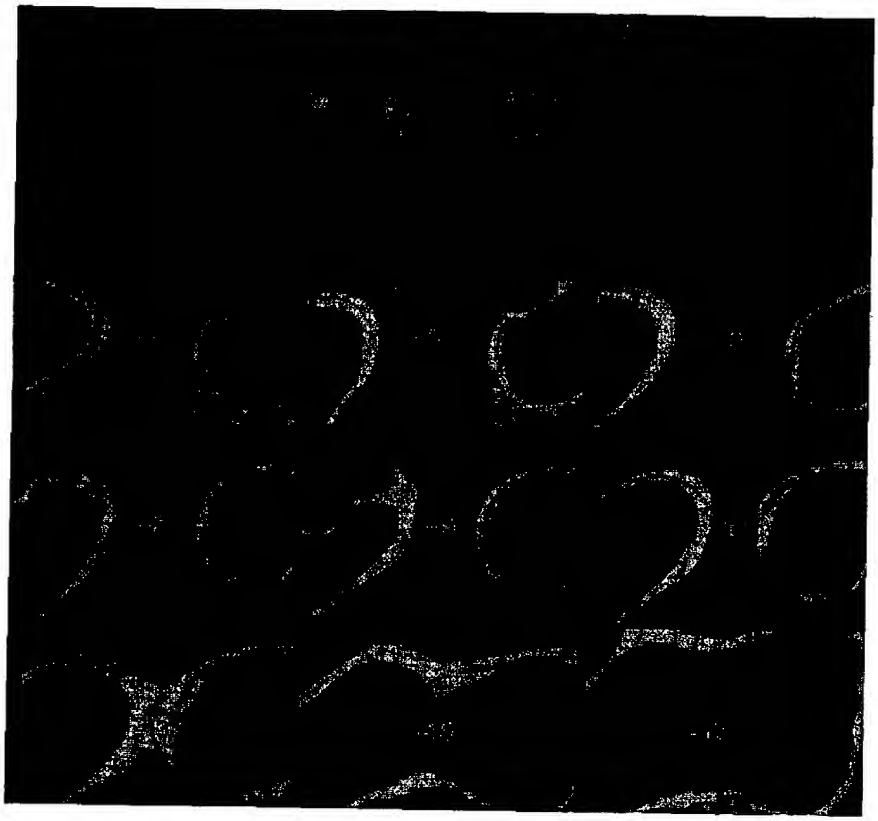

FIG. 7. A visualization of new bone formation in animals treated with intermittent PTH. A reconstruction of the bone formation events around a small fenestration site is presented. Although the site is designated as a fenestration site, it is interesting to note the appearance of eroded surface on the upper surface, representing remnants of previous osteoclast action (arrows) and thus a possible perforation site. A series of 10 μm sections of calcein-labeled cancellous bone from an Ovx animal, treated with intermittent PTH for 3 weeks, was photographed. Every other section (20 μm apart) in the series is displayed in the figure. The remaining bone sample, after serial sectioning, was processed for scanning electron microscopy and displayed at the top of the graph. Sections are arranged by the numbers of sections distal to the bone surface shown in SEM picture.

a complex pattern of changes in bone formation and resorption. After a rapid elevation in bone formation and a non-significant transient increase in bone resorption, increased bone formation persisted and subsequently resulted in thicker trabecular plates, improved cancellous bone volume, and greater mechanical competency, without an increase in trabecular number. Our results indicate that PTH primarily acts by adding bone to existing trabecular plates and in support of the findings of Qi et al.[33] that treatment will be less effective if bone loss has progressed to the stage where trabecular number is already severely depleted.

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cytokine Redundancy 4 IL-11

The Role of IL-11 in Hematopoiesis as Revealed by a Targeted Mutation of Its Receptor

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Key Words. *Interleukin 11 · Receptor · Hematopoiesis · Thrombopoiesis · Progenitors · Gene targeting*

ABSTRACT

Interleukin 11 (IL-11) is a pleiotropic growth factor with several actions in common with members of the IL-6 family. IL-11 utilizes a specific receptor chain encoded by two genes, IL-11Ra, which is expressed in hematopoietic and other tissues and, IL-11Ra2, which has a restricted pattern of expression. The actions of IL-11 in the hematopoietic compartment include support of multilineage and committed progenitors contributing to myeloid, erythroid, megakaryocyte, and lymphoid lineages. IL-11 demonstrates a prominent thrombopoietic activity which is being evaluated in clinical trials. In contrast to the multiple in vitro and in vivo effects of IL-11, mice with a targeted mutation of the IL-11Ra gene (IL-11Ra-/-) did not exhibit an overt hematological phenotype. Generation of a null phenotype was confirmed by independent assays. The numbers of progenitor cells of various lineages as well as their terminally differentiated progeny were undisturbed in the IL-11Ra-/- mice. In addition, the mutant mice were able to respond appropriately to increased demand in situations of hematopoietic stress. This study has highlighted the growth factor redundancy operative in the hematopoietic compartment, and in addition, has served to identify a critical action of IL-11 in nonhematopoietic organs. *Stem Cells 1998;16(suppl 2):53-65*

INTRODUCTION

Interleukin 11 (IL-11) was originally identified as a stromal-cell-derived growth factor able to stimulate the proliferation of a murine plasmacytoma cell line [1, 2]. In addition to its action on the B-cell lineage, its ability to support the growth of multilineage progenitors was soon recognized. Subsequently, the action of IL-11 on multiple hematopoietic lineages was demonstrated and reviewed [3-7]. However, the effects of IL-11 are not restricted to hematopoiesis. IL-11 has effects on varied organs; for example, it aids recovery of spermatogenesis after chemotherapy [8], ameliorates the effect on the intestinal epithelium of cytotoxic injury and inflammatory bowel disease [9, 10], and is a possible mediator of bronchial reactivity [11]. Consistent with the known metabolic functions of IL-11, the cDNA was also independently cloned by *Kawashima et al.* based on its ability to inhibit adipogenesis in 3T3-L1 cells [12]. The nonhematopoietic actions of IL-11 have been recently reviewed [7].

The pleiotropic actions of IL-11 on the hematopoietic compartment and other organ systems are shared with those of other members of the IL-6 family of growth factors, including leukemia

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inhibitory factor (LIF), oncostatin-M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin (CT). The overlapping function of these growth factors is, in part, explained by shared receptor components [13-15]. For instance, the IL-6 family of cytokines share gp130 as a signal transduction unit [14, 16]. Several of these molecules also utilize the LIF receptor (LIFR) as a component of their receptor complex; also, specific receptor chains exist for OSM, IL-6, and CNTF [14, 16, 17]. Work in our laboratory led to cloning of the cDNAs encoding the murine and human IL-11-specific receptor subunit (α-chain) and the demonstration that the expression of this receptor component alone confers low-affinity IL-11 binding. Coexpression with gp130 results in high-affinity binding of IL-11 and the capacity for signal transduction [18, 19]. We have also previously described the presence of two loci for the murine IL-11 receptor α-chain, IL-11Ra and IL-11Ra2 [20, 21]. While transcripts corresponding to the IL-11Ra locus are expressed widely in hematopoietic and other organs, those from the IL-11Ra2 locus are detected only at relatively low levels in the testis, lymph node, and thymus [20, 21]. Furthermore, the IL-11Ra2 locus is not present in all mouse strains; for example, SJL/J and DBA/2J are two murine strains that contain only the ubiquitously expressed IL-11Ra gene, while 129/Sv, C57BL/6, and BALB/cBy also contain the IL-11Ra2 gene [20]. The cDNAs from the two loci exhibit 99% nucleotide identity in the coding exons, but contain different 5' untranslated regions [20, 21]. This divergence permits discrimination between transcripts from the two loci using reverse-transcriptase polymerase chain reaction (RT-PCR).

Despite the varied actions of IL-11 within the hematopoietic compartment, its principal physiological role remains unclear. The generation of genetically modified animals is increasingly used to ascertain the in vivo importance of the molecules of interest. We sought to understand the essential functions of IL-11 in vivo by generation of mice with a targeted null mutation of the IL-11Ra gene [22]. Here we describe the role of IL-11 in hematopoiesis as revealed by analysis of the IL-11Ra receptor null (IL-11Ra-/-) mice.

IL-11 and Thrombopoiesis

It was initially observed by *Paul et al.* and later confirmed by other investigators that IL-11 acting alone had no megakaryocyte colony-forming activity in vitro but could augment this activity of cytokines such as IL-3, stem cell factor (SCF; or kit-ligand), and thrombopoietin (TPO) [1, 23-28]. IL-11 alone and in synergy with IL-3 also induces features of megakaryocyte maturation including increased ploidy, size, and production of acetylcholinesterase [24, 26]. In vivo experiments with IL-11 have demonstrated both an enhancement of megakaryocyte progenitors and an increase in platelet production (see below). Although megakaryocytes have been shown to express IL-11 receptors [29], it has been speculated that the action of IL-11 on megakaryocytes is mediated through additional growth factors. This question has been addressed, in part, by examining the effect of IL-11 on highly purified target populations in an attempt to eliminate contaminating stromal elements which could act as a source of other factors. *Teramura et al.* reported that IL-11 acting alone was able to induce maturation markers in purified human CD41-positive cells [25]. As human megakaryocytes have the potential to produce IL-6 and to express receptors for IL-6 [30], it was possible that the action of IL-11 on purified megakaryocytes was mediated through IL-6. However, the addition of anti-IL-6 antibody to the culture system was shown not to abrogate the response to IL-11 [25]. As the enormous potential of TPO to support megakaryopoiesis became apparent, *Kaushansky et al.* examined the possibility that TPO might mediate the thrombopoietic response to other growth factors, including IL-11 [28]. The addition of soluble c-mpl receptor as a TPO antagonist abrogated IL-6, IL-11, or SCF-induced in vitro megakaryocyte colony formation from unfractionated bone marrow cells, suggesting a dependence on TPO [28]. The generation of mice deficient in TPO or c-mpl permitted this issue to be addressed in vivo [31-33]. Administration of IL-6, IL-11, SCF, or LIF to these

mice resulted in a platelet increment [34, 35]: thus, through demonstrating in vivo, these growth factors can act independently of TPO.

Support for the action of IL-11 in megakaryocytopoiesis has also come from the analysis of IL-11 in animal models. Administration of IL-11 to normal and splenectomized mice elevated platelet counts in both groups and was associated with an increment in the number of megakaryocytes and progenitors in bone marrow and spleen [36-38]. IL-11 also induced megakaryocyte maturation in vivo; analysis of bone marrow megakaryocytes revealed an increase in ploidy [36, 37]. Mice in which IL-11 was overexpressed demonstrated a persistent elevation of platelets associated with an increment in megakaryocytes [39-41]. IL-11 also possesses megakaryopoietic and thrombopoietic action in monkeys [42] and dogs [43]. Administration of IL-11 to humans also results in an elevation of platelet count, increased megakaryocyte numbers in the marrow with a higher fraction in cell cycle, and a shift to higher ploidy [44-46].

While the action of IL-11 on platelets is clear, there are conflicting data regarding the relationship between circulating levels of IL-11 and the platelet/megakaryocyte mass. Elevated IL-11 levels were observed in patients with thrombocytopenia along with consequent increased megakaryocyte mass, as a result of immune platelet destruction [47]. In contrast, TPO levels are not elevated in situations of immune destruction as reviewed [48, 49]. IL-11 levels were also noted to be elevated in patients with thrombocytopenia and decreased megakaryocyte mass due to myelosuppression, with a significant inverse correlation between endogenous IL-11 levels and circulating platelet counts during recovery [47]. This finding, however, has not been consistently observed [50]. It is interesting that in mpl-null mice with platelet counts 10%-15% of normal, no elevation of IL-11 levels has been observed [35]; thus, unlike with TPO, there is no simple relationship between levels of serum IL-11 and total mass of cells of the megakaryocyte lineage.

Given the prominent activity of IL-11 on this lineage, it was surprising to find that the IL-11Ra-/- mice had no perturbation of platelet numbers (Fig. 1). There was no difference in platelet counts for wild-type, heterozygous, or IL-11Ra-/- mice. These findings were also reflected in the bone marrow and spleen. Shown in Figure 2 are representative histology sections from sternum and spleen of wild-type and IL-11Ra-/- mice. There was no difference in the number of megakaryocytes nor in the composition of other hematopoietic lineages. Also, no difference was noted in the number of megakaryocyte progenitors [22]. This is in contrast to the situation in IL-6 null mice, which display a normal platelet count but have an ~50% reduction in megakaryocyte colony-forming cells (MK-CFC) [51]. The role of IL-11 in situations of hematopoietic stress was also examined. We have explored this in

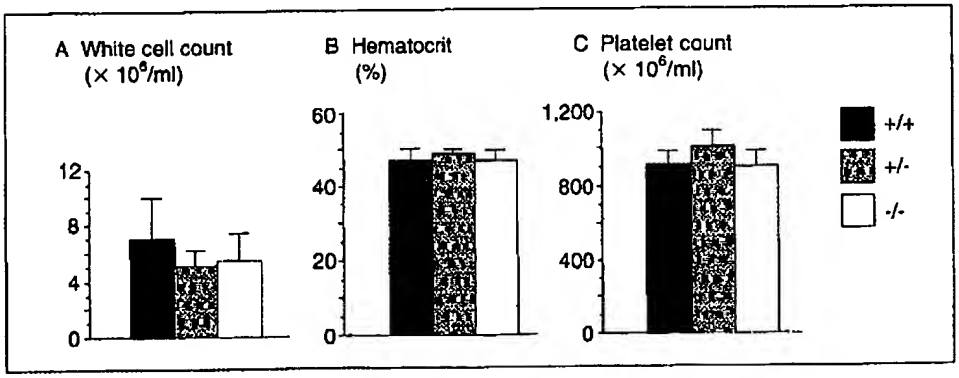

Figure 1. Full blood count profile of IL-11Ra-/-, heterozygous (IL-11Ra+/-) and wild-type (IL-11Ra+/+) mice (n = 6 animals per group; mean ± SD).

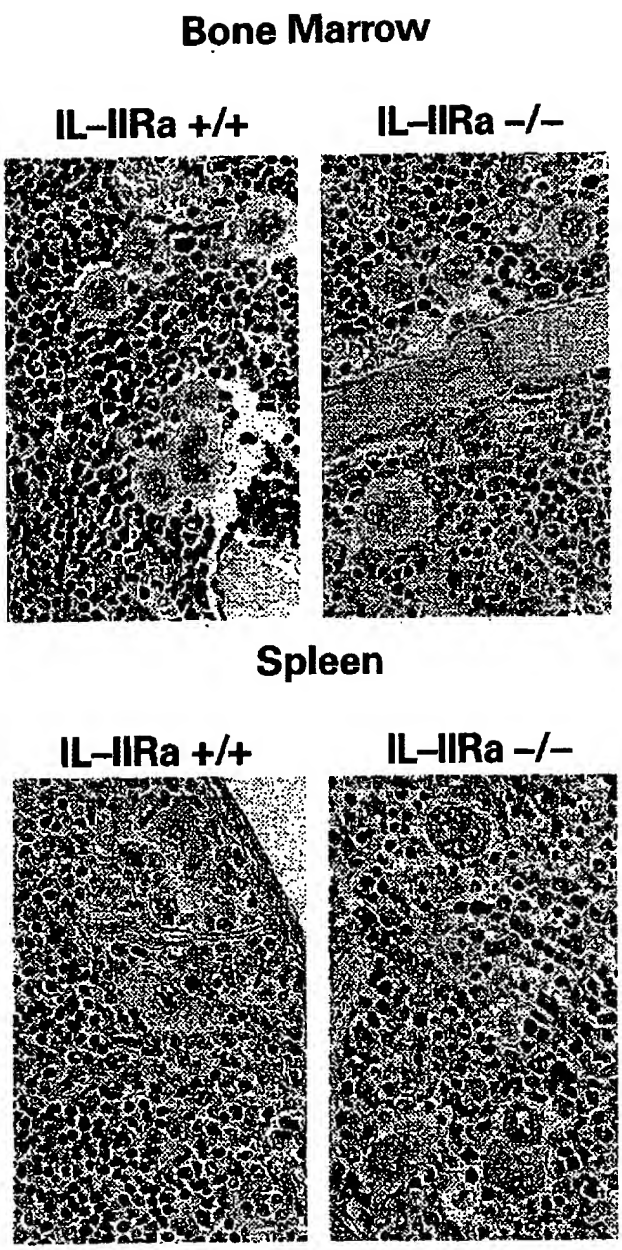

Figure 2. Representative sternal and spleen histology from IL-11Ra+/+ and IL-11Ra-/- mice. The number and morphology of megakaryocytes were normal in the IL-11Ra-/- mice and were assessed in twenty high-power fields (magnification × 400) by an investigator blinded as to the genotype. A minimum of nine mice per genotype were examined.

models of myelosuppression secondary to 5-FU and immune platelet destruction (by administration of antiplatelet serum leading to a 90% fall in platelets). In both of these situations, the rate and amplitude of platelet recovery in IL-11Ra-/- mice was similar to the wild-type [22]; thus baseline megakaryocyte/platelet levels and responses to thrombocytopenic stress occurred normally in these animals in the absence of IL-11 signaling.

IL-11 and Erythropoiesis

IL-11 has been shown to influence multiple stages of erythropoiesis. While IL-11 in the presence of erythropoietin (EPO) is inadequate to maintain erythropoiesis in long-term marrow cultures [52], IL-11 shows synergy with SCF in expanding BFU-E potential of such cultures at least four- to fivefold [53]. IL-11 stimulates BFU-E when combined with IL-3, even in the absence of EPO [54, 55] and with other growth factors such as SCF and GM-CSF [56]. IL-11 also supports the maturation of colony-forming unit-erythroid (CFU-E) [54]. The effect of IL-11 on erythroid progenitors was not abrogated by the addition of antibodies to SCF, IL-3, or GM-CSF, thus suggesting a direct effect of IL-11 on human and murine erythroid cells, at least in the in vitro system [55]. Studies in mice have demonstrated that IL-11 can stimulate erythroid progenitors (BFU-E, CFU-E) and that this potential is further amplified by concurrent administration with SCF [57, 58]. The effect of IL-11 alone on the erythroid compartment also translates to a dose-dependent increase in reticulocytes [58]. In contrast to the

stimulatory activity of IL-11 on erythroid cells, IL-11 induced a mild anemia in mice [58]. Such an effect is also seen in dogs [43], monkeys [42], and humans [45], and is thought to be due to volume expansion [59].

Despite the evidence implicating IL-11 in erythropoiesis, in the IL-11Ra-/- mice, the hematocrit and reticulocyte count as well as the numbers of erythroid progenitors were all normal (Fig. 1) [22]. In keeping with these results, the IL-6 null mice also show a normal hematocrit (but with expansion of erythroid progenitors) but exhibit a 100% mortality in response to hemolysis induced by phenylhydrazine. A similar experiment was performed in the IL-11Ra-/- mice; however, their response was completely normal. This was true both in terms of the degree of hemolysis produced using the same dose as was lethal in the IL-6 null mice and of the kinetics of erythroid recovery. Therefore, although IL-11 can influence multiple stages of erythroid development, IL-11 signaling in the adult is not critical for maintaining basal erythropoiesis nor in generating a normal erythroid response to hemolytic stress.

IL-11 and Myelopoiesis

Several investigators have shown that IL-11 shows little activity in stimulating myeloid colonies in in vitro cell cultures but enhances the growth of a range of multipotential and committed myeloid progenitors (Mix-CFC, granulocyte-macrophage [GM]-CFC, M-CFC) in combination with other growth factors such as IL-3, SCF, GM-CSF [52, 60-65], IL-4 [61, 66], IL-7, IL-12 [67-69], IL-13 [66], flt3-ligand [70], and TPO [71]. The expansion of myeloid progenitors and peripheral blood neutrophilia is also seen with in vivo administration [42, 57, 72]. However, a neutrophilic response is not consistently seen in all experimental models. For example, mice in which IL-11 expression was enforced showed >20-fold elevation in myeloid progenitors but had unchanged leukocyte levels [40]. Similarly, neutrophilia was not noted in dog studies [43] nor in a human phase I trial [45].

The analysis of the myeloid compartment revealed no abnormality in the IL-11Ra-/- mice. There were normal neutrophil numbers, and there was no perturbation in myeloid progenitors as ascertained by in vitro assays [22]. The analysis of hematopoietic progenitors was also extended to a study of their capacity for self-generation. Clone-transfer experiments were performed that involved the physical isolation of individual colonies supported by SCF and the replating of these cells in secondary cultures also stimulated by SCF. Shown in Table 1 are results for colonies recloned from both wild-type and IL-11Ra-/- mice. There was no deficit in the recloning potential from IL-11Ra-/- mice.

Table 1. Recloning potential of SCF-stimulated colonies from IL-11Ra-/- bone marrow cells

Genotype	Number of primary colonies examined	Percent recloning mean (range)	Number of secondary clones generated (mean ± SD)
+/+ ($n = 7$)	239	12 (3%-38%)	68 ± 42
-/- ($n = 7$)	214	13 (6%-20%)	108 ± 79

Primary colonies from bone marrow cells were grown in SCF (100 ng/ml) and isolated, prepared as a single cell suspension, and recultured in agar stimulated by SCF. The number of secondary clones arising from each primary colony was enumerated. Primary colonies included those with blast-cell morphology and multicentric colonies, with no difference between wild-type (+/+) and IL-11Ra-/- cells. Results are pooled from the analysis of seven mice of each genotype.

IL-11 and Stem Cells

While there is convincing evidence for the expansion of committed progenitors by IL-11, the activity of this growth factor on the primitive stem cell population remains unresolved. Addition of IL-11 to human and murine long-term marrow cultures has been observed to increase the nonadherent cell population which contained increased numbers of multilineage and committed progenitors such as colony-forming unit-spleen (CFU-S) day 12, Mix-CFC, GM-CFC, and BFU-E [52, 73]. The proliferative effects have been shown to be due to the entry of a quiescent (G_0) population into active cell cycle as well as the shortening of cell cycle time [64, 74]. However, IL-11 had no significant effect on the high-proliferative potential (HPP)-CFC and, in fact, decreased the frequency of human long-term culture-initiating cells (LTC-IC) and murine long-term reconstituting stem cells [73]. Similar results were demonstrated in short-term (six days) liquid cultures of bone marrow cells taken two days after treatment with 5-FU. Cells cultured with IL-11 as the sole stimulus were incapable of generating primitive day 28 cobblestone area-forming cells (CAFC) and did not contain stem cells capable of competitive repopulation [75]. These results implied that IL-11 enhanced the commitment of stem cells into a multipotential progenitor compartment. Similarly, *Holyoake et al.* described a 50-fold amplification of multipotential progenitors after short-term (six days) culture of unfractionated BM cells with SCF and IL-11 [76]. The ex vivo expanded cell population contributed to an accelerated short-term engraftment and also sustained quartenary bone marrow transplant recipients as opposed to unmanipulated marrow [76]. Despite an amplification of HPP-CFC and LPP-CFC with induction of active cell cycling, other workers have described a defect in the engraftment potential of IL-11-treated cells [77, 78]. A direct comparison, however, cannot be made between these transplantation studies since they differed in experimental design. Moreover, it is difficult to attribute either the expansion or attenuation of long-term reconstitution capacity to IL-11, since it was only one component of differing cytokine combinations. In models of chronic IL-11 overexposure, bone marrow cells overexpressing IL-11 resulted in accelerated engraftment of tertiary recipients [39, 41]. Therefore, continued exposure of stem cells to IL-11 did not lead to stem cell extinguishment. IL-11 also acts to cause mobilization of stem cells/progenitor cells into peripheral blood [79].

Results to date suggest, as with the other hematopoietic compartments, that the stem cell compartment is probably also preserved intact in the IL-11Ra-/- mice. Levels of CFU-S day 12 were normal; however, the definitive assay for stem cell function is hematopoietic reconstitution. These results should be contrasted with those for the LIF null and the IL-6 null mice. The LIF-/- mice have an ~60% and ~90% decrease in bone marrow and spleen CFU-S day 12 content, respectively, as well as a reduction in GM-CFC and erythroid progenitors [80]. The IL-6-/- mice also showed a reduction in CFU-S day 12 as

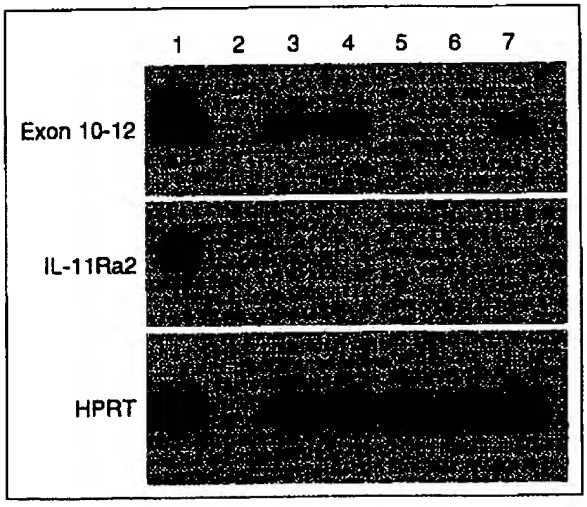

Figure 3. RT-PCR confirming absence of IL-11Ra transcript in fetal liver cells from IL-11Ra-/- mice (lanes 5 and 6) and demonstrating absence of expression of IL-11Ra2 transcript in fetal liver cells from IL-11Ra-/- mice as well as the control IL-11Ra+/- mice (lanes 3,4, and 7). RT-PCR products encoding the deleted exons of the IL-11Ra gene (upper panel), transcripts specific to the IL-11Ra2 gene (middle panel) and hypoxanthine phosphoribosyltransferase (HPRT) gene (lower panel) were examined by Southern hybridization with radiolabeled internal oligonucleotide probes. Controls used were testis which expresses both transcripts (positive control, lane 1) and negative control (no cDNA, lane 2).

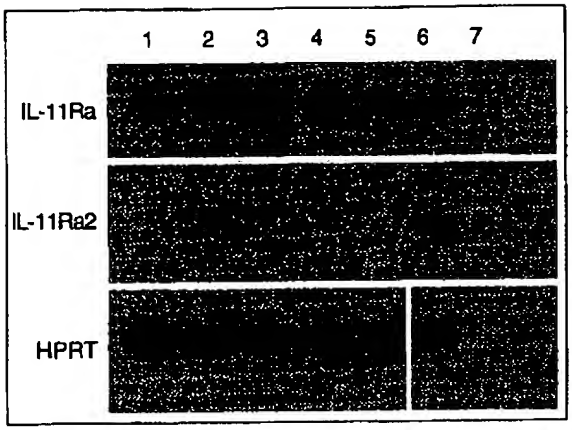

Figure 4. RT-PCR demonstrating expression of IL-11Ra transcript and lack of expression of IL-11Ra2 transcript in embryonic day 9.5 yolk sac tissue. The cDNA samples were wild-type yolk sacs (lanes 1 and 4) and wild-type embryo (lane 3), and controls used were yolk sac (lane 2) and embryo (lane 5) from mice with a targeted mutation of the scl gene [81], testis which expresses both transcripts (positive control; lane 6), and negative control (no cDNA, lane 7). RT-PCR products specific to the IL-11Ra gene (upper panel), transcripts specific to the IL-11Ra2 gene (middle panel), and HPRT gene (lower panel) were examined by Southern hybridization with radiolabeled internal oligonucleotide probes.

well as in long-term reconstitution potential [51]. This implies a progenitor/stem cell subset that is critically dependent on LIF or IL-6. Such a cell population has not yet been identified for IL-11. The overall physiological significance of this defect in the LIF null or IL-6 null mice is unclear, however, since despite reduced levels of CFU-S, the LIF null and the IL-6 null mice have normal numbers of peripheral blood leucocytes.

We have also analyzed the role of IL-11 signaling in earlier stages of hematopoiesis. Experiments were performed using fetal livers and yolk sac tissue. An RT-PCR-based assay was used to determine the genotype and examine gene expression in these tissues. As shown in the Figure 3 upper panel, lanes 5 and 6 represent fetal liver cells from IL-11Ra-/- as they lack expression of exons 10-12 which were deleted by the targeting strategy. For comparison, lanes 3, 4, and 7 are from heterozygous pups. A transcript for IL-11Ra2 was never identified in hematopoietic cells (middle panel), an observation in common with hematopoietic cells from adult animals. Similarly, no expression of the IL-11Ra2 gene was detected in day 9.5 yolk sac tissues and embryos (Fig. 4). Expression of IL-11Ra gene was present in wild-type yolk sac tissues and embryos and also in those mutant for the scl gene [81] (included as a further control; Fig. 4), but as expected, was not seen in IL-11Ra-/- tissues (data not shown).

The progenitor cell content of fetal livers and yolk sacs from mutant and wild-type mice was assessed by in vitro clonal cultures. These assays were scored by an investigator blinded as to the genotype of the cells being examined. As shown in Figure 5, the numbers of GM-CFC, BFU-E, and MK-CFC in fetal livers from mutant and control (IL-11Ra+/-) mice were comparable. GM-CFC and BFU-E progenitors were also quantified from yolk sacs. This involved dissecting the individual yolk sacs, preparing a single-cell suspension, and stimulating the cells with the cytokine combination indicated. Again, the number of progenitor cells was similar for the two genotypes.

IL-11 and Lymphopoiesis

In common with its effect on hematopoietic progenitors, IL-11 supports the generation of lymphoid progenitors only in the presence of other cytokines. For example, in combination with IL-4 or SCF, IL-11 enhanced the generation of B cells in primary cultures of bone marrow cells [69, 82-84] and together with IL-7, flt3-ligand, or SCF, IL-11 stimulated the growth of uncommitted progenitors and their differentiation into the B lineage [70, 85-87]. The action of IL-11 in promoting B-cell differentiation (increased DNA synthesis, immunoglobulin secretion, and augmentation of sheep red blood cell-specific antibody responses) was found to be dependent on the presence of accessory T cells but independent of coproduction of IL-6 [1, 88, 89]. Our results have shown that the production of immunoglobulin isotypes as well as the production of specific antibodies after primary and secondary antigen challenge was maintained in the IL-11Ra-/- mice [22]. However, since the predominant action

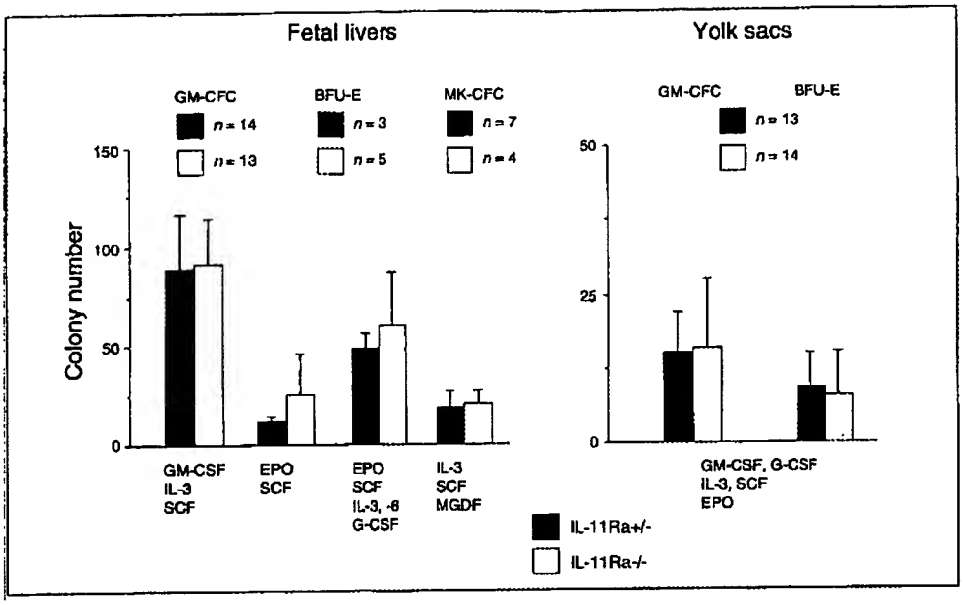

Figure 5. Granulocyte-macrophage (GM-CFC), BFU-E, and megakaryocyte progenitors (MK-CFC) in fetal livers and yolk sacs of IL-11Ra-/- mice. Results are mean ± standard deviation of total colony numbers obtained from replicate cultures. Cultures of fetal livers contained 50,000 cells. GM-CFC were enumerated from agar cultures stimulated with GM-CSF, IL-3, and SCF. BFU-E were from methylcellulose cultures stimulated either with EPO and SCF or with EPO, SCF, IL-3, IL-6, and G-CSF. MK-CFC were from agar cultures stimulated with IL-3, SCF, megakaryocyte growth and development factor (MGDF), and stained with acetylcholinesterase. Results are expressed per 50,000 fetal liver cells. Yolk sac cultures were all performed in methylcellulose and with the cells from individual yolk sacs being equally divided into quadruplicate cultures and stimulated with GM-CSF, G-CSF, IL-3, SCF, and EPO. Results are expressed as number of colonies per yolk sac. Cells were stimulated with final concentrations of GM-CSF 10 ng/ml, IL-3 10 ng/ml, SCF 100 ng/ml, MGDF 200 ng/ml, EPO 2 U/ml, IL-6 100 ng/ml, and G-CSF 10 ng/ml. (n = the number of independent samples examined.)

of IL-11 in this compartment is in combination with other growth factors, a phenotype may be revealed in genetic crosses with mice lacking other molecules operative in this lineage.

Lack of Hematopoietic Phenotype Is Not Due to Compensation by IL-11Ra2

Given the overwhelming body of data implicating IL-11 in stem cell regulation, thrombopoiesis, erythropoiesis, and myeloid cell differentiation, the lack of hematopoietic phenotype in the IL-11Ra-/- mice was very surprising. Explanations for a lack of a detectable effect on the hematopoietic compartment included the possible failure to generate a germline IL-11Ra null phenotype and/or compensation from the untargeted IL-11Ra2 gene. Experiments were therefore performed to directly exclude these possibilities. First, Southern analysis confirmed the desired mutation of the IL-11Ra locus. Second, Northern analysis demonstrated an absence of IL-11Ra (and IL-11Ra2) transcripts, and this was confirmed by RT-PCR on bone marrow cells and on colonies derived from those cells [22]. Moreover, bone marrow cells from IL-11Ra-/- mice did not respond to IL-11 in in vitro cultures, while bone marrow cells from wild-type mice responded normally [22]. We have also confirmed the inability of IL-11Ra-/- mice to respond to administered IL-11. As shown in Table 2, in vivo administration of IL-11 caused the expected ~1.5-fold increment in platelet count in the wild-type mice. Such a response was not observed in the IL-11Ra-/- mice. No consistent changes in either the white cell count or hematocrit were noted in either genotype. These experiments documented both the inability to respond to IL-11 and the lack of compensation from the IL-11Ra2 gene.

Table 2. IL-11 administration does not increase platelet count in IL-11Ra-/- mice

Mice genotype		Stimulus	Platelet count × 10^6/ml Day 0	Day 6
B-463	+/+	NS	844	836
B-483	+/+	NS	604	736
B-531	+/+	NS	760	876
B-513	-/-	NS	944	844
B-515	-/-	NS	832	940
B-516	-/-	NS	848	1,000
B-534	+/+	IL-11	980	1,516*
B-525	+/+	IL-11	912	1,652*
B-528	+/+	IL-11	912	1,328*
B-487	-/-	IL-11	1068	1,160
B-488	-/-	IL-11	936	876
B-489	-/-	IL-11	800	908

IL-11 (Genetics Institute) was administered s.c. at 250 μg/kg/day in 200 μl of normal saline (NS)/0.1% BSA, in two divided doses for six days. Controls were injected with NS/BSA. Platelet count was measured before and after stimulus administration.

The increment in platelet count following IL-11 administration was significant for only the wild-type (IL-11Ra+/+) mice (*$p = 0.02$).

Conclusion

The study of murine models made genetically deficient in growth factors or their cognate receptors has provided important insights into the in vivo role of these molecules. For example, analysis of phenotypes resulting from targeted mutation of TPO [31] or its receptor c-mpl [32, 34], or the mutation of GM-CSF [90, 91] and its signal transduction molecule, the common β subunit [92, 93] have provided complementary data regarding the role of these ligands and their receptors. Our results imply that although IL-11 exhibits multiple actions in the hematopoietic compartment, these are well compensated in the mice with a targeted mutation of the receptor. Is this because of the untargeted second gene or due to expression of an as-yet-unidentified receptor component? Both possibilities seem unlikely since the mutant mice have been shown to be unable to respond to IL-11 in vitro as well as in vivo. The hematopoiesis-stimulating activity of IL-11, identified in in vitro and in vivo studies, has been tested in various models of myelosuppression: bone marrow transplant, chemotherapy, irradiation, and combined chemotherapy and irradiation [7]. The favorable effect of IL-11 on hematopoietic recovery, observed in various animal models, has led to its evaluation in several human clinical trials. An attenuation of post-chemotherapy thrombocytopenia and a reduction in platelet transfusion requirements has been observed, as well as an acceleration of the recovery of neutrophils when combined with granulocyte colony-stimulating factor [45, 46, 94-96]. Thus, although hematopoiesis is undisturbed in mice lacking the IL-11 receptor, this does not detract from the potential clinical utility of IL-11. The targeting study has, however, served to highlight the degree of redundancy that operates within the hematopoietic system.

IL-11 also has pleiotropic effects outside the hematopoietic compartment. Our receptor targeting strategy has also served to identify an additional role for IL-11 as a critically important mediator in reproductive physiology, since the IL-11Ra-/- female mice are infertile [22, 97]. Another potentially important role for

this growth factor is its enhancement of gastrointestinal epithelial repair after cytotoxic insult and in inflammatory bowel disease. The combination of this action and its ability to stimulate thrombopoiesis suggests an application in the context of cytotoxic therapy with deleterious effects on both organs; thus, IL-11 is an exciting growth factor whose contribution to normal development and potential therapeutic uses is beginning to be unraveled.

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